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(54) Title: NEW IMMUNOPROTECTIVE INFLUENZA ANTIGEN AND ITS USE IN VACCINATION

(57) Abstract

The present invention relates to an influenza antigen, comprising a fusion product of at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and a presenting carrier, which may be a presenting (poly)peptide or a non-peptidic structure, such as glycans, peptide mimetics, synthetic polymers. The invention further relates to a vaccine against influenza, comprising at least an antigen of the invention, optionally in the presence of one or more excipients. The invention also relates to use of the antigen, a method for preparing the antigen and acceptor cells expressing the antigen.

NEW IMMUNOPROTECTIVE INFLUENZA ANTIGEN AND
ITS USE IN VACCINATION

The present invention relates to new
immunoprotective influenza antigens, which are non-
existent in nature. The invention further relates to the
use of the antigens for vaccination and to vaccines
5 containing them, as well as to methods for preparing the
antigens.

Influenza is caused by an RNA virus of the
myxovirus group. Influenza viruses can be classified into
three types (A, B and C), based on antigenic differences
10 in the nucleoprotein and the matrix protein. Type A and B
influenza viruses each contain 8 RNA segments, while type
C only has 7 RNA segments. Influenza A is most important
and is very pathogenic for man, as well as for animals,
for example pigs and horses. Type B influenza causes
15 disease in humans. Influenza C is less severe and has
been isolated from humans and pigs. The virus is
transmitted through the air, mainly in droplets expelled
during coughing and sneezing. The influenza viruses cause
an infection of the respiratory tract, that is usually
20 accompanied with coughing, high fever and myalgia.
Although an influenza infection does not often lead to
the death of the infected individual, the morbidity can
be severe. As a consequence thereof influenza epidemics
may lead to substantial economic loss. Furthermore,
25 influenza infection can be more dangerous for certain
groups of individuals, such as those having suffered from
a heart attack, CARA patients or the elderly. A vaccine
against influenza is therefore highly desirable.

The influenza A virus contains in its membrane
30 two highly immunogenic, but very variable proteins, the
hemagglutinin and the neuraminidase. Due to the
variability of these two proteins a broad spectrum, long
lasting vaccine against influenza A has so far not been
developed. The influenza vaccine commonly used, has to be
35 adapted almost every year to follow the antigenic drift
of the virus. In these circumstances the vaccine can
protect about 80% of the immunized persons. When more

drastic changes occur in the virus, known as antigenic shift, the vaccine is no longer protective.

It is therefore the object of the present invention to provide a new immunoprotective antigen for
5 use in vaccines which is not based on the rapidly changing hemagglutinin and/or neuraminidase and which therefore lacks the disadvantages of these known antigens and vaccines based thereon.

In the research that led to the present
10 invention it was found that well conserved membrane proteins of influenza other than hemagglutinin and neuraminidase can be used for eliciting protection. Particularly useful for this approach is the membrane protein M2.

15 M2 mRNA is encoded by RNA segment 7 of the influenza A virus. It is encoded by a spliced mRNA (Lamb et al., 1981). Like the hemagglutinin and the neuraminidase, the M2 protein is an integral membrane protein of the influenza A virus. But the protein is much
20 smaller, only 97 amino acids long. 24 amino acids at the amino terminus are exposed outside the membrane surface, 19 amino acids span the lipid bilayer, while the remaining 54 residues are located on the cytoplasmic side of the membrane (Lamb et al., 1985).

25 The M2 protein is abundantly expressed at the cell surface of influenza A infected cells (Lamb et al., 1985). The protein is also found in the membrane of the virus particle itself, but in much smaller quantities, 14 to 68 molecules of M2 per virion (Zebedee and Lamb,
30 1988). The M2 protein is posttranslationally modified by the addition of a palmitic acid on cysteine at position 50 (Sugrue et al., 1990).

The M2 protein is a homotetramer composed of two disulfide-linked dimers, which are held together by
35 noncovalent interactions (Sugrue and Hay, 1991). By site-directed mutagenesis, Holsinger and Lamb (1991) demonstrated that the cysteine residue at position 17 and 19 are involved in disulfide bridge formation. Only

cysteine at position 17 is present in all viruses
analyzed, therefore it seems likely that this is the most
important residue. In the virus strains where cysteine 19
is also present, it is not known whether a second
5 disulfide bridge is formed in the same dimer (already
linked by Cys 17 - Cys 17) or with the other dimer.
      By aligning the sequences of M2 proteins,
isolated from different human strains of influenza A
virus, a striking conservation of the extracellular part
10 of the M2 protein, became evident (table 1). Since the
first human influenza A strain isolated in 1933, A/WS/33
(H1N1), until the most recently sequenced virus
A/Guangdong/39/89 (H3N2), no amino acid change has been
observed in the extracellular domain of the M2 protein.
15 Two virus strains do not fit in this conserved pattern,
A/PR/8/34 (H1N1), which shows one amino acid change, and
A/Fort Monmouth/1/47 (H1N1), which shows three amino acid
differences. These two strains probably represent side
branches in the evolutionary tree.
20   Table 1 gives an overview of the amino acid
sequences of the extracellular domain of the influenza A
M2 protein of the virus strains A/WSN/33 (Markushin et
al. (1988)), A/PR/8/34 (Allen et al. (1980), Winter and
Fields (1980)), A/WS/33, A/Fort Warren/1/50,
25 A/Singapore/1/57 and A/Port Chalmers/1/73 (all described
by Zebedee and Lamb (1989)), A/Udorn/72 (Lamb and Lai
(1981)), A/Leningrad/134/57 (Klimov et al. (1992)), A/Ann
Arbor/6/60 (Cox et al. (1988)), A/Bangkok/1/79 (Ortin et
al. (1983)), A/New York/83 (Belshe et al. (1988)), A/Fort
30 Monmouth/1/47 (EMBL U02084), A/USSR/90/77 (EMBL X53029)
and A/Guangdong/39/89 (EMBL L 18999).

4

Table 1

Amino acid sequence of the extracellular domain of the M2 protein

Strain	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A/WS/33 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/WSN/33 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/PR/8/34 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	[Gly]	Ser	Ser	Asp
A/Fort Monmouth/1/47 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	[Thr	Lys]	Asn	Glu	Trp	[Glu]	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Fort Warren/1/50 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/USSR/90/77 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Singapore/1/57 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Leningrad/134/57 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Ann Arbor/6/60 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Udorn/72 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Port Chalmers/1/73 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Bangkok/1/79 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/NY/83 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Guangdong/39/89 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp

It was anticipated by the present inventors
that the conserved character of this type of membrane
proteins could make them good candidates for vaccine
development. In principle, the protective capacity of
5 anti-M2 antibodies is already known. Experimental data
demonstrated that a monoclonal antibody directed against
the extracellular part of the M2 protein (14C2) can
diminish the spread of the virus, although the
infectivity of the virus in vitro was not reduced
10 (Zebedee and Lamb, 1988). Furthermore it was demonstrated
that passively administered monoclonal antibody (14C2)
could inhibit viral multiplication in the lungs of mice
(Treanor et al., 1990). Both approaches rely on the
administration of anti-M2 antibodies. However, the
15 passive administration of monoclonal antibodies as a
means of defense against infection is preferably avoided
because of the immunogenicity of heterologous
immunoglobulins which, upon repeated administration, can
lead to the clearing of the antibodies from the body and
20 thus to a reduction of the efficacy of the treatment.
Even homologous antibodies can elicit anti-idiotype
antibodies. Furthermore, it was found that humans
infected with the virus do have anti-M2 antibodies but
these do not protect against infection, (either their
25 concentration or their nature are not sufficient to
confer efficacy). This makes it unlikely that passive
administration of anti-M2 antibodies is suitable for use
in humans. It also teaches away from trying to develop
vaccines for humans based on this antigen.
30   Recently, protection of mice against an
infection with homologous or heterologous virus was
described (Slepushkin et al., 1995). These authors used a
formulation of incomplete Freund's adjuvant and a
membrane extract of Sf9 cells expressing the complete M2
35 protein for immunizations. However, this approach is also
not suitable for vaccination of humans because it relies
on the use of the exceptionally potent Freund's adjuvant
which is prohibited in humans.

In summary, use of antibodies for providing protection against influenza is preferably to be avoided. Moreover, it is unlikely that prophylactic treatment with antibodies will be effective in humans. Immunization with
5 complete M2 protein in humans as described is not realistic because it relies on incomplete Freund's adjuvant which cannot be used in humans, and is counter-indicated in higher animals.

It is thus the object of the present invention
10 to provide for an alternative influenza antigen that is sufficiently immunoprotective against a broad spectrum of influenza strains and is not dependent on Freund's adjuvant, such that it can be used in human beings.

According to the invention it has now been
15 found that it is possible to prepare such a novel antigen that does not exist in nature. For this the extracellular part of a conserved influenza membrane protein or a functional fragment thereof is fused to a presenting carrier, for example a (poly)peptide. The conserved
20 influenza membrane protein is for example the well conserved, extracellular part of the M2 protein. The membrane protein is preferably genetically fused to a presenting (poly)peptide as the presenting carrier, which (poly)peptide stabilizes the extracellular part and
25 surprisingly potentiates the immunogenicity of the fusion product thus obtained. It is thought that the presenting (poly)peptide brings the extracellular part into its wild type structure, thus presenting the antigen in a form that is also found on the virus and on the infected
30 cells.

A 'functional fragment of the conserved influenza membrane protein' is a fragment that is capable of eliciting a statistically significant higher immunoprotection when administered in an immunoprotective
35 dose to test members of a species than is found in control members of the same species not receiving the functional fragment.

In one embodiment of the invention the 23 amino acid extracellular part of the M2 protein is fused to the amino terminus of the human Hepatitis B virus core protein. In this way the wild type structure of the M2
5 protein in viral particles and on infected cells, where the free N-terminus extends in the extracellular environment, is mimicked.

Alternative presenting (poly)peptides are multiple C3d domains (Dempsey et al., 1996), tetanus
10 toxin fragment C or yeast Ty particles. 'Presenting (poly)peptides' are intended to encompass every stretch of amino acid(s) that can present the extracellular part, in a substantially wild type form, towards the environment.

15 Alternatively, the presenting carrier can be a non-peptidic structure, such as glycans, polyethylene glycols, peptide mimetics, synthetic polymers, etc..

After expression of the novel antigen in a suitable acceptor cell, it can be used either as such
20 (depending on the acceptor cell), as part of a membrane fragment or in isolated form.

The term 'presenting carrier' is used to indicate all types of presenting molecule, both (poly)peptides and others.

25 It will be clear for the person skilled in the art that a gene construct, comprising the coding information for the antigen and the presenting (poly)peptide, can not only be used to prepare the new antigen, as described above, but that it can also be
30 used, optionally in the presence of suitable transcription and/or translation regulatory sequences, in a DNA vaccine, or in vaccinia based vaccine constructions.

A presenting (poly)peptide can be incorporated
35 into the fusion product in a single copy or in multiple copies. The third complement protein fragment d (C3d) is preferably used in more copies, preferably 3 or more.

In a preferred embodiment of the invention the fusion product further may comprise an additional peptide at an appropriate internal site (Schödel et al., 1992) or C-terminal (Borisova et al., 1989). This additional

5 peptide is intended to further increase the protective capacity of the antigen, and may for example be a T helper cell epitope or a cytotoxic T cell epitope.

The antigen of the invention is obtainable by preparing a gene construct comprising a coding sequence

10 for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and optionally the coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription

15 and/or translation and/or secretion regulatory sequences, bringing this gene construct in a suitable acceptor cell, effecting expression of the gene construct in the acceptor cell and optionally isolating the antigen from the acceptor cell or its culture medium.

20 The requirement for transcription and/or translation and/or secretion regulatory sequences depends on whether the gene is to be integrated into a vector or whether integration in the genome of the acceptor cell is at a position already providing these signals.

25 The coding sequence for a presenting (poly)peptide is only present when the fusion product is a fusion between the antigen and a peptidic structure and if it is desirable to directly link the two structures in the DNA construct. In all other instances, the presenting

30 carrier may be added to the antigen in a different manner.

The suitable acceptor cell can be selected for example, from E. coli, Lactococcus lactis, Lactobacillus plantarum, yeast (e.g. Pichia pastoris), insect cells

35 (e.g. Sf9), mammalian cells (e.g. Vero cells) and the like. In the case of L. lactis the antigen need not be isolated but the engineered bacteria can be used directly for intranasal or oral use.

The invention further relates to vaccines that comprise at least the antigen of the invention. This antigen can be in isolated form or being part of a membrane fragment or being expressed on the acceptor

5 cell. The antigen of the invention can be used together with suitable excipients. The person skilled in the art of vaccine design will be capable of selecting suitable excipients. Guidance may for example be found in Methods in molecular medicine: Vaccine Protocols (1996). Eds.

10 Robinson, A., Farrar, G.H. and Wiblin, C.N. Humana Press, Totowa, New Jersey, USA.

The antigens of the invention may be used alone or in combination with one or more other influenza antigens, such as neuraminidase, hemagglutinin or native

15 M2.

Furthermore, the invention relates to the use of the antigens in the preparation of a vaccine against influenza. The vaccines can be direct vaccines, i.e. vaccines containing the fusion products or indirect, DNA

20 vaccines. The latter are vaccines, comprising the fusion cDNA under the regulation of a eukaryotic promoter that can function in the recipient. The actual antigen is then produced in the recipient of the vaccine.

The vaccines of the invention are intended both

25 for use in humans and in animals, for example pigs and horses of which it is known that they are infected by influenza A.

A similar approach as described here for preparing novel fusion antigens of influenza A can be

30 adopted to prepare similar fusion antigens and vaccines containing the fusion antigens or DNA encoding the fusion antigens for influenza B and C.

The invention also relates to a method of preparing the antigens, comprising the steps of:

35   a) preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a

presenting (poly)peptide operably linked thereto,
optionally in the presence of suitable transcription
and/or translation and/or secretion regulatory sequences,

      b) bringing this gene construct in a suitable

5 acceptor cell,

      c) effecting expression of the gene construct
in the acceptor cell, and

      d) optionally isolating the antigen from the
acceptor cell or its culture medium.

10   The invention will be further illustrated by
the following example, that is in no way intended to
limit the invention. The example describes in detail the
preparation of fusion proteins of M2 sequence with
various presenting (poly)peptides and the use thereof in

15 immunization. Instead of M2 and the presenting carriers
described here, the skilled person will be capable of
choosing another conserved influenza membrane protein and
other presenting carriers.

      In the example reference is made to the

20 following figures:

      Figure 1 : Construction of pATIPM2ml.
E1 and E2 = first and second exon of the influenza M2
protein,
M2e = extracellular part of the M2 protein,

25 M2t = transmembrane part; and
M2c = cytoplasmic tail.
Bold line = vector.
  (a) removal of the intron out of the m2 gene,
  (b) introduction of a BclI site between the

30   extracellular part and the transmembrane domain of
      the M2 protein,
  (c) nucleotide and amino acid sequence of the
      extracellular part of the M2 protein of A/PR/8/34.

      Figure 2 : Construction of pIPM2hB2Mm2s2.

35 ori = origin of replication,
  cat = chloramphenicol acetyltransferase,
  bla = ß-lactamase,
  lpp = lipoprotein,

hB2M = human ß₂-microglobulin,

ompa-ss = signal sequence of the outer membrane protein A

of <u>E. coli</u>,

ssDNA = single-stranded DNA,

5 M2e = extracellular part of the M2 protein.

(a) : Construction flow scheme,

(b) : Details of key sequences.

Figure 3 : Construction of pPLcIPM2HBcm.

ori = origin of replication,

10 cat = chloramphenicol acetyltransferase,

bla = ß-lactamase,

HBc = hepatitis B core,

ssDNA = single-stranded DNA,

M2e = extracellular part of the M2 protein.

15 (a) : Plasmid construction flow scheme,

(b) : Sequence around the introduced BamHI restriction

site in the hepatitis B core gene,

(c) : Details of key sequences.

Figure 4 : Analysis of the soluble fraction,

20 corresponding to 150 µl original culture, of strain

MC1061[pcI857] containing the plasmids pPLc245 (control),

pPLcA1 (expression of HBc) or pPLcIPM2HBcm (expression of

IPM2HBcm) respectively, on a SDS 12.5% PAGE. After the

electrophoresis the gel was stained with Coomassie

25 brilliant blue.

MW = molecular weight marker,

NI = not induced culture,

I = induced culture.

Figure 5 : Analysis of the soluble fraction,

30 corresponding to 150 µl original culture, of strain

MC1061[pcI857] transformed with pPLc245 (control), pPLcA1

(expression of HBc) or pPLcIPM2HBcm (expression of

IPM2HBcm) respectively, as in figure 4. After

electrophoresis, the relevant proteins were revealed by a

35 Western blotting experiment. Detection with (A) a

monoclonal antibody against HBc and (B) a monoclonal

antibody specific for the extracellular part of the M2

protein.

MW = molecular weight marker,

NI = not induced culture,

I = induced culture.

Figure 6 : Sequence of the amino terminus of
5 the M2 protein compared to the amino terminus of
IPM2HBcm, as experimentally determined. Sequence of
A/Udorn/72 (Lamb and Zebedee, 1985).

Figure 7 : Soluble fractions of strain
MC1061[pc1857] transformed with pPLc245 (control), pPLcA
10 1 (expression of HBc) or pPLcIPM2HBcm (expression of
IPM2HBcm), respectively, analyzed in a native state by
means of a dot blot. Detection with (A) a monoclonal
antibody against HBc and (B) a monoclonal antibody
specific for the extracellular part of the M2 protein.
15 NI = not induced culture,

I = induced culture.

Figure 8 : Overview of (A1) rectal temperature,
(A2) weight and (B) survival of the mice vaccinated with
IPM2HBcm after a lethal challenge with 5 LD_{50} m.a.
20 A/PR/8/34. The statistical significance was calculated by
the Fisher's exact test. Mice immunized with different
doses of antigen were compared to the control group. The
following results were obtained: for 50 μg IPM2HBcm
$p < 0.001$; for 10 μg $p < 0.005$ and for the 5 μg dose $p < 0.05$.
25 Figure 8C shows the survival of the mice vaccinated
intraperitoneally with IPM2HBcm, and IM2HBcm,
respectively, after a lethal challenge with 30 HAU X-47.
Figure 8D shows the survival of the mice vaccinated
intranasally with IPM2HBcm, and IM2HBcm, respectively,
30 after a lethal challenge with 30 HAU X-47.

Figure 9 : Analysis of the serum samples of the
four set ups reported in figure 8. The pre-immune serum
(a), the serum taken after the first (b), after the
second (c) and after the third (d) immunization and the
35 serum taken after challenge (e) were initially diluted
1/50. The consecutive dilution steps were 1/3. The
plotted absorbance is a corrected value obtained as
described in Results, Analysis of the serum samples.

Figure 10 : Construction of pPLcIM2HBcm.

ori = origin of replication,

cat = chloramphenicol acetyltransferase,

bla = ß-lactamase,

5 M2e = extracellular part of the M2 protein,

HBc = hepatitis B core.

Figure 11 : Analysis of the soluble fraction,
containing 5 µg HBc or I(P)M2HBcm (as determined in an
ELISA (see Materials and methods)), of strain MC1061

10 [pcI857] containing respectively the plasmids pPLc245
(control), pPLcA1 (expression of HBc), pPLcIPM2HBcm
(expression of the fusion protein IPM2HBcm with the
extracellular part of the M2 protein derived from
A/PR/8/34) or pPLcIM2HBcm (expression of IM2HBcm,

15 containing the more universal M2 sequence) on a SDS 12.5%
PAGE-gel.

MW = molecular weight marker,

NI = not induced,

I = induced culture.

20   Figure 12 : Analysis of the soluble fraction,
containing 2.5 µg HBc or I(P)M2HBcm (as determined in an
ELISA (see Materials and methods)), of strain MC1061
[pcI857] containing respectively the plasmids pPLc245
(control), pPLcA1 (expression of HBc), pPLcIPM2HBcm

25 (expression of IPM2HBcm) or pPLcIM2HBcm (expression of
IM2HBcm) on a Western blot (see Materials and methods).
Detection with (A) a monoclonal antibody directed against
HBc and (B) a monoclonal antibody specific for the
extracellular part of the M2 protein.

30 MW = molecular weight marker,

NI = not induced,

I = induced culture.

        Figure 13 : Overview of the oligonucleotides
used for PCR amplification of hbc and i(p)m2hbc. 's' or

35 'a' following the name of the oligonucleotide stands for
the use of these primers in the sense (s) or anti-sense
(a) orientation. The boxed sequence indicates the changed
Leu codons.

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Figure 14 : Overview of the construction of hbc
and m2hbc fusions in vectors for <u>L. lactis</u>.
ori = origin of replication for <u>E. coli</u>,
ori(+) = origin of replication for <u>L. lactis</u>,
5 ermA and ermM = erythromycin resistance genes,
P1 = <u>L. lactis</u> promoter,
bla = ß-lactamase,
HBc = hepatitis B core,
M2e = extracellular part of the M2 protein,
10 usp45-ss = signal sequence of usp45,
mIL2 = murine interleukin 2 and
mIL6 = murine interleukin 6.

Figure 15 : Analysis of the expression of
Hepatitis B core (HBc) and M2-HBc fusion proteins in a
15 Western blot. An equivalent of 10^9 <u>L. lactis</u> bacteria of
strain MG1363 containing respectively pTREX1 (control),
pT1HBc, pT1HBcIL2, pT1HBcIL6 (expression of HBc alone or
in combination with mIL2 or mIL6, respectively),
pT1PM2HBc, pT1PM2HBcIL2, pT1PM2HBcIL6 (expression of
20 IPM2HBcm alone or in combination with mIL2 or mIL6,
respectively), pT1M2HBc, pT1M2HBcIL2, pT1M2HBcIL6
(expression of IM2HBcm alone or in combination with mIL2
or mIL6, respectively), was analyzed in a SDS 12.5% PAGE-
gel. The first antibody, p-anti-HBc (Dako Corporation,
25 Carpinteria, CA., USA) was diluted 5000 times. The bound
antibodies were detected with a 1/2000 dilution of the
polyclonal anti-rabbit IgG labeled with alkaline
phosphatase (Southern Biotechnology Associates,
Birmingham, AL., USA). I(P)M2HBc stands for either
30 IPM2HBcm or IM2HBcm.
MW = molecular weight marker,
C = control and
- = expression of the antigen alone.

Figure 16 : Analysis of the expression of M2-
35 HBc fusion proteins in a Western blot. An equivalent of 2
to 3x10^9 <u>L. lactis</u> bacteria of strain MG1363 containing
respectively pT1HBc (control), pT1PM2HBc, pT1PM2LHBc
(expression of IPM2HBcm), pT1M2HBc, pT1M2LHBc (expression

of IM2HBcm), was separated on a SDS 12.5% PAGE-gel. The
fusion proteins were detected with an IgG fraction of a
polyclonal mouse anti-M2e antibody (see Materials and
methods). The bound antibodies were detected with a
5 1/2000 dilution of the alkaline phosphatase conjugated
polyclonal anti-mouse IgG (γ-chain specific) (Southern
Biotechnology Associates, Birmingham, AL., USA).
MW= molecular weight marker,
C = control,
10 E = leucine codons optimal for use in E. coli, and
L = leucine codons optimal for use in L. lactis.
These are the plasmids pT1PM2LHBc and pT1M2LHBc,
respectively. I(P)M2HBc stands for either IPM2HBcm or
IM2HBcm.

15   Figure 17 : Overview of the oligonucleotides
used for PCR amplification of the extracellular part of
the M2 protein and C3d.
's' or 'a' following the code name of the oligonucleotide
stands for the use of these primers in the sense (s) or
20 anti-sense (a) orientation. The boxed region indicates
the changed Leu codons.
        Figure 18 : Overview of the construction of
m2c3d3 fusions in L. lactis.
ori = origin of replication for E. coli,
25 ori(+) = origin of replication for L. lactis,
ermA and ermM = erythromycin resistance genes,
P1 = L. lactis promoter,
bla = ß-lactamase,
M2e = extracellular part of the M2 protein,
30 usp45-ss = signal sequence of usp45,
spaX = anchor sequence derived from Staphylococcus aureus
protein A,
C3d = complement protein 3 fragment d, and
mIL6 = murine interleukin 6.
35   Figure 19 : Overview of the oligonucleotides
used for PCR amplification of ttfc and m2ttfc.
's' or 'a' following the name of the oligonucleotide
stands for the use of these primers in the sense (s) or

anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

Figure 20 : Overview of the construction of m2ttfc in vectors for L. lactis.

5   ori = origin of replication for E. coli,

ori(+) = origin of replication for L. lactis,

ermM and ermμ = erythromycin resistance genes,

P1 = L. lactis promoter,

bla = ß-lactamase,

10 TTFC = tetanus toxin fragment C,

M2e = extracellular part of the M2 protein,

usp45-ss = signal sequence of usp45,

mIL2 = murine interleukin 2, and

mIL6 = murine interleukin 6.

15   Figure 21 : Analysis of the expression of IPM2TTFC fusion protein in a Western blot. An equivalent of 10^9 L. lactis bacteria of strain MG1363 containing respectively pT1TT (control), pT1PM2LTT (expression of IPM2TT), pT1PM2LTTIL2 (expression of IPM2TT in

20 combination with mIL2) or pT1PM2LTTIL6 (expression of IPM2TT in combination with mIL6), was analyzed in a SDS 10% PAGE-gel. The first antibody, an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and methods) was diluted 2500 times. The bound antibodies

25 were detected with a 1/2000 dilution of the polyclonal anti-mouse IgG labeled with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL., USA). 30 mg 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo., USA), was dissolved in 10 ml methanol.

30 Afterwards 40 ml PBS, pH 7.4 and 150 μl H_2O_2 was added.

MW = molecular weight marker,

- = expression of the antigen alone,

mIL2 = expression of the antigen in combination with mIL2,

35 mIL6 = expression of the antigen in combination with mIL6.

Figur 22 : Primer set used for PCR amplification of the secretion signal of the gp67 baculovirus protein.

Figure 23 : Primer set used for PCR
5 amplification of the extracellular part of the M2 protein during construction of the sgpM2C3d3 fusion.

Figure 24 : Construction of the baculovirus transfer vector pACsgpM2C3d3.

bla = ß-lactamase,
10 bold grey line = baculovirus homology region,
C3d = complement protein 3 fragment d,
M2e = extracellular part of the M2 protein,
ori = origin of replication,
phP = baculovirus polyhedrin promoter, and
15 sgp67 = secretion signal of the gp67 baculovirus protein.

Figure 25 : Detail of nucleotide and amino acid key sequences of the sgpM2C3d3 fusion.

C3d = complement protein 3 fragment d,
M2e = extracellular part of the M2 protein, and
20 sgp67 = secretion signal of the gp67 baculovirus protein.

Figure 26 : Analysis of recombinant AcNPV/sgpM2C3d3 baculovirus by PCR amplification of the polyhedrin locus (primers TTTACTGTTTTCGTAACAGTTTTG and CAACAACGCACAGAATCTAG). Control reactions were performed
25 with the parental transfer vector pACsgpM2C3d3 and with wild type AcNPV baculovirus.

M = DNA size markers.

Figure 27 : Expression of secreted M2C3d3 by
Sf9 insect cells infected with recombinant
30 AcNPV/sgpM2C3d3 baculovirus as demonstrated by Western analysis (10% PAGE-gel) of harvested supernatant. Supernatant from mock infected cells or obtained after infection with wild type AcNPV baculovirus are included as a control.
35 MW = molecular weight markers.

Figure 28 : Overview of the survival of mice after a lethal challenge with 5 LD_{50} m.a. X47. Mice

vaccinated with 3x10 μg IM2HBcm are compared with
passively immunized mice (P).

      Figure 29 : Overview of the DNA vaccination
constructs.

5 RT = reverse transcriptase

PCMV = cytomegalovirus promoter

bla = ß-lactamase

npt = neomycin resistance.

      Figure 30 : Expression in HEKT cells analyzed
10 on a Western blot. The first antibody (paM2 (see
Materials and Methods)) was diluted 2000 times. The bound
anti-M2 antibodies were detected with an alkaline
phosphatase labelled anti-mouse IgG.

MW = molecular weight marker

15 M2 = M2 protein expressed in insect cells

  1 = pCDNA3

  2 = pCIM2

  3 = pCIM2HBcm

  4 = pCIP3M2HBcm.

20   **Figure 31** : Antibody response against the M2
protein analyzed in an ELISA.

      A. Microtiterplates were coated with periplasm
containing hB2M or IPM2hB2M respectively (see Materials
and Methods).

25   B. Microtiterplates coated with M2 protein
expressed in insect cells (see Materials and Methods).

      The following abbreviations will be used:

1 LD_{50}	:	lethal dose, the viral challenge required
30		to kill half of the population of infected
		mice
BCIP	:	5-bromo-4-chloro-3-indolylphosphate
bp	:	base pair(s)
CIP	:	calf intestine phosphatase
35 C3d	:	complement protein 3 fragment d
DEA	:	diethylamine
HAU	:	hemagglutination units
hB2M	:	human ß2-microglobulin

19

HBc	:	Hepatitis B core protein
IM2HBcm	:	universal influenza A M2 protein fragment fused to HBc
IPM2hB2Mm	:	influenza A M2 protein fragment (from A/PR/8/34) fused to hB2M
IPM2HBc	:	influenza A M2 protein fragment (from A/PR/8/34), fused to HBc, containing four additional amino acids between the first methionine and the start of the extracellular part of the M2 protein
IPM2HBcm	:	influenza A M2 protein fragment (from A/PR/8/34) fused to HBc
IPTG	:	isopropyl-ß-D-thiogalactoside
m.a.	:	mouse adapted
M2C3d3	:	universal influenza M2 fragment fused to three copies of C3d
cM2C3d3	:	cytoplasmic form of M2C3d3
sM2C3d3	:	secreted form of M2C3d3
sM2C3d3X	:	form of M2C3d3 covalently attached to the cell wall
MES	:	2-(N-morpholino)ethanesulphonic acid
MPLA	:	monophosphoryl lipid A
NBT	:	nitro blue tetrazolium
OmpA-ss	:	signal sequence of the outer membrane protein A
PCR	:	polymerase chain reaction
SDS-PAGE	:	sodium dodecylsulfate polyacrylamide gel electrophoresis
TDM	:	trehalose dicorynomycolate
phP	:	baculovirus polyhedrin promoter
sgp67	:	secretion signal of the baculovirus gp67 protein

EXAMPLE

INTRODUCTION

This example demonstrates the preparation of
various fusion antigens based on the influenza A virus M2

protein. The M2 fragment was fused to the amino terminus of various presenting carriers.

MATERIALS AND METHODS

5 1. Bacterial strains and plasmids

All plasmid constructions, made for expression in Escherichia coli, were performed in strain MC 1061 (hsdR mcrB araD139Δ(araABC-leu)7697 ΔlacX74 galU galK rpsL thi (Casadaban and Cohen, 1980) because of high

10 efficiency of transformation. The first transformation after mutagenesis was performed in WK6λmutS (Δ(lac-proAB), galE, strA, mutS::Tn10/lacIq, ZΔM15, proA·B·; Zell and Fritz, 1987). Expression studies of human ß$_2$-microglobulin and derivatives were performed in E. coli

15 strain C3000 (Hfr, sup·, thi(λ·)). Expression studies of the Hepatitis B core protein and derivatives were carried out in MC1061 [pcI857].

pcI857 was described in Remaut et al., 1983b. A derivative of this plasmid pcI857K1 was described in

20 Steidler et al., 1994.

The plasmid p714 (Parker and Wiley, 1989) was a kind gift of Dr. K. Parker and the plasmid pPLcA1 (Nassal, 1988) of Dr. M. Nassal. The plasmid pPLc245 was described in Remaut et al., 1983a.

25 For the constructions and expressions in Lactococcus lactis strain MG1363 (Gasson, 1983) was used. The vector for constitutive expression in L. lactis, pTREX1 (Wells and Schofield, 1996) was a generous gift from Dr. K. Schofield. The plasmid pL2MIL2, for the

30 expression of interleukin 2, is described in Steidler et al., 1995. An analogous plasmid for the expression of interleukin 6, pL2MIL6, is described in Steidler et al., 1996.

The vector pSG5.C3d.YL (Dempsey et al., 1996)

35 is a gift from Dr. Fearon.

The baculovirus transfer vector pACGP67A (Pharmingen, San Diego, CA, USA) contains a modified segment of the baculovirus genome, including the

polyhedrin promoter followed by the secretion signal derived from the gp67 baculovirus protein and a cloning site for the insertion of a foreign gene sequence. It is constructed to allow integration into the baculovirus
5 genome (or modified version thereof) by homologous recombination. The resulting recombinant baculovirus is capable of expressing the gene of interest from the polyhedrin promoter as a secreted protein by cleavage of the gp67 secretion signal.
10

2. Virus

Influenza virus A/PR/8/34 (H1N1) was adapted to mice by several lung passages. After adaptation, the virus was grown in eggs (Kendal et al, 1982) and purified
15 over a sucrose gradient. The titer [(hemagglutination units (HAU) (Hirst, 1941; Kendal et al, 1982)] and the lethality in mice were determined. For m. a. A/PR/8/34, 1 LD_{50} corresponded to 10 HAU present in 50 μl.

Influenza strain X-47 (H3N2) (Baez et al.,
20 1980) was used in experiments for heterologous challenge. This strain was adapted to mice by several lung passages.

3. Animals

Female Balb/c mice were purchased from Charles
25 River Wiga (Sulzfeld, Germany). The mice were used at the age of 6 to 7 weeks.

4. Antibodies

The monoclonal mouse antibody directed to the
30 Hepatitis B core protein was a kind gift from Dr. Sc. H. Claeys (Bloedtransfusiecentrum, Leuven).

A mouse monoclonal antibody specific for the human $ß_2$-microglobulin was purchased from Boehringer (Mannheim, Germany).
35 Alkaline phosphatase conjugated antibodies specific for mouse IgG or mouse IgG (γ chain specific) were bought from Sigma Chemical Co. (St. Louis, Mo., USA).

5. Growth media

E. coli was grown in LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) unless mentioned otherwise. The minimal M9 medium (Miller, 1972),
5   supplemented with 0.2% casamino acids, was used in experiments when the expressed proteins were secreted into the growth medium and had to be purified.

M17 growth medium (Difco Laboratories, Detroit, MI, USA)) supplemented with 0.5% glucose (GM 17) was used
10 for culturing L. lactis. Erythromycin was used at a concentration of 5 μg/ml (medium GM17E). L. lactis was grown at 28°C without shaking.

The hybridomas and the myeloma cells were grown in RPMI 1640 (Gibco BRL, Bethesda, Md., USA) supplemented
15 with 10% fetal calf serum, 0.3 mg/ml L-glutamine, 0.4 mM sodium pyruvate, 100 u/ml penicillin and 100 ng/ml streptomycin.

Sf9 insect cells were grown in TC100 medium (Gibco BRL, Bethesda, MD, USA) supplemented with 10%
20 fetal calf serum, 100 U/ml penicillin and 100 ng/ml streptomycin.

6. Adjuvants

For the first immunization Ribi adjuvant (Ribi
25 Immunochem Research Inc., Hamilton, MT, USA) was used. A complete dose of Ribi adjuvant contains 50 μg MPLA (monophosphoryl lipid A), 50 μg TDM (trehalose dicorynomycolate), 2% squalene and 0.01% Tween 80.

For the second and third immunization MPLA
30 (Ribi Immunochem Research Inc., Hamilton, MT, USA) was used alone or mixed with an equal quantity of adjuvant peptide (Sigma Chemical Co., St. Louis, Mo., USA).

7. DNA manipulations

35   Restriction enzymes, DNA polymerases, T4 polynucleotide kinase and T4 DNA ligase (Boehringer, Mannheim, Germany; Gibco BRL, Bethesda, Md. USA, or New England Biolabs, Beverly, MA, USA) were used as

seconds at 72°C. A total of thirty amplification rounds
were performed. The control reactions did not contain
oligonucleotides. Three different concentration of MgSO₄
were used, 2, 3 and 4 mM. The PCR reaction that produced
5 a significant amount of the expected fragment under the
most stringent conditions (lowest Mg²⁺ concentration and
highest T$_m$) was used for further cloning.

The C3d3 fragment was amplified from
pSG5.C3d.YL with the oligonucleotides C3ds and C3da using
10 Pwo DNA Polymerase (Boehringer, Mannheim, Germany). The
annealing temperature was set at 60°C and the synthesis
was performed for 2 min at 72°C.

Amplification of the baculovirus gp67 secretion
signal was done with Taq polymerase (Boehringer Mannheim,
15 Germany) from pACGP67A using the primers GP67s en GP67a.
A total of 25 cycli were performed with synthesis at 72°C
for 1 min.

9. Ligation

20   The ligations for L. lactis were performed with
Ready-To-Go™ T4 DNA Ligase (Pharmacia Biotech, Uppsala,
Sweden). After incubation for 1h at 20°C, the mixture was
extracted with phenol (Life Technologies, Paisley, UK)
and chloroform/iso-amyl alcohol (24/1). The DNA was
25 precipitated with see-DNA (Amersham International,
Buckinghamshire, UK). The complete resuspended pellet was
used for electroporation (Wells et al., 1993).

10. Protein purification media

30   All chromatography media were purchased from
Pharmacia Biotech (Uppsala,Sweden), except CF11
cellulose, which was purchased from Whatman International
Ltd. (Maidstone, UK).

35 11. Protein gel

Protein samples were analyzed by SDS-
polyacrylamide gel electrophoresis (SDS-PAGE) according
to Laemmli, 1970. After electrophoresis, the proteins

25

were fixed with 10% trichloroacetic acid and stained with
0.05% Coomassie brilliant blue R-250 in destain. Excess
dye was removed by incubating the gel in destain (30%
methanol - 7% acetic acid). The gel was soaked in 40%
5 ethanol before it was dried between two sheets of
permeable cellophane.

12. Western blot and dot blot

        For immunological characterization, proteins
10 were electrophoretically transferred from a SDS-PAGE-gel
onto a nitrocellulose membrane (pore diameter 0.45 µm,
Schleicher & Schuell, Dassal, Germany) with a dry
blotting apparatus (Plexi-labo, Gent, Belgium). The
filter was blocked for at least 2h in PBS pH 7.4 (14.5 mM
15 phosphate buffer pH 7.4 - 150 mM NaCl) with 2.5% skim
milk powder and 0.1 % Triton X-100 (blocking buffer).
Incubation with the primary antibody, diluted in blocking
buffer, was carried out at room temperature for 30 to 60
min. Excess of unbound antibody was removed by three
20 washings with blocking buffer. The bound antibodies were
detected with an alkaline phosphatase conjugated antibody
of the appropriate specificity. Subsequently, the filter
was washed two times with PBS pH 7.4 - 0.1% Triton X-100.
A third washing step was carried out with substrate
25 buffer (100 mM Tris-HCl pH 9.5 - 100 mM NaCl - 5 mM
$MgCl_2$). The filter was then incubated in substrate buffer
with 165 µg/ml nitro blue tetrazolium (NBT) and 165 µg/ml
5-bromo-4-chloro-3-indolylphosphate (BCIP) until a clear
signal appeared. The blot was finally washed thoroughly
30 with tap water and dried.
        The dot blot analysis was carried out in a
similar way as the Western blot, except that the proteins
were not transferred through electrophoresis, but by
filtering the samples through a nitrocellulose membrane.
35

13. ELISA

        In every ELISA a 0.1 % casein solution was used
for blocking and for making the dilutions of the

26

antibodies used. The stock solution of casein (2.5%) was prepared as follows: 6.25 g casein powder was dissolved in 200 ml 300 mM NaOH by overnight stirring at 37°C. Then the pH was adjusted to 7.0 by adding 2N HCl. The final

5 volume was brought to 250 ml (Nunc bulletin no. 7, December 1989). Sodium azide (0.02%) was added as a preservative.

Different ELISA's were developed to determine the concentration of Hepatitis B core or human ß2-

10 microglobulin fusion proteins. Microtiter plates (type II F96 maxisorp Nunc A/S, Roskilde, Denmark) were coated for 1.5 h at room temperature or overnight at 4°C with a 1/2 dilution series of samples containing IPM2HBcm or IPM2hB2Mm. On the same plate, a 1/2 dilution series of

15 purified HBc or hB2M, respectively, starting from 2 μg/ml, was used as a standard. Between every incubation step, the plates were washed twice with tap water and once with PBS, pH 7.4 - 0.05% Triton X-100, except that after blocking, the plates were not washed. The

20 microtiter plates were blocked with 0.1% casein solution for 2h at room temperature or at 4°C overnight. As primary antibody we used mouse anti-HBc or mouse anti-hB2M, respectively. The bound antibodies were detected with an alkaline phosphatase labelled anti-mouse IgG (γ

25 chain specific) antibody. The incubation with antibody solution was carried out at room temperature for 1.5 h. Finally the microtiter plates were incubated for 1 h with substrate buffer (10% diethanolamine - 0.5 mM $MgCl_2$ - 0.02% NaN_3 pH 9.8) containing 1 mg/ml p-nitrophenyl

30 phosphate. The absorbance was measured at 405 nm and the wave length of 490 nm was used for normalization.

14. Preparation of polyclonal anti-M2

All mice, which had been immunized with

35 IPM2HBcm and had survived the lethal challenge with m.a. A/PR/8/34 influenza A virus (see results, immunization) were anaesthetized with 250 μl 25 mg/ml tribromoethanol (injected i.p.) and blood samples were taken by heart

puncture. The serum was isolated as described
hereinbelow. The crude serum gave a high background in
Western blot, therefore an IgG fraction was prepared. The
crude serum was filtered through a 0.45 μm filter
5 (Millipore Millex-HV, Millipore, Bedford, MA, USA) and
diluted 10 times in loading buffer (PBS - 10 mM EDTA, pH
8). This mixture was loaded on an equilibrated Protein G
Sepharose 4 Fast Flow column (φ = 1 cm, h = 8 cm), The
bound IgG molecules were eluted with 100 mM glycine-HCl,
10 pH 2.7. Fractions of 1 ml were collected in tubes
containing 50 μl 1 M Tris-HCl pH 9.5 to bring the pH to
neutral.

        The quantity of anti-M2 antibodies in the
pooled peak fractions was 2.6 μg/ml. This was determined
15 in an ELISA, comparable to the detection of anti-M2
antibodies in the serum of immunized mice. Mouse
monoclonal anti-human ß2-microglobulin (Cymbus
Bioscience, Southampton, UK) was used as a standard.

20 <u>15. Serum preparation</u>
        Five blood samples were taken from every mouse:
the pre-immune serum (a), the serum taken after the first
(b), after the second (c) and after the third (d)
immunization, and the serum taken after challenge (e).
25 This blood was incubated for 30 min at 37°C. The samples
were then placed on ice for at least 1 hour and
centrifuged two times 5 min at 16000 g in a
microcentrifuge. The serum was isolated.
        Equal volumes of sera obtained from different
30 mice were pooled for the analysis of antibody production.

<u>16. RT-PCR</u>
        Allantoic fluid of A/Ann Arbor/6/60 (215 HAU)
was incubated in AMV buffer (Boehringer, Mannheim,
35 Germany) at 65°C for 30 min. 1/20 of this mixture was
used for the reverse transcriptase (RT) reaction. Too
this vRNA (genomic <u>v</u>iral <u>RNA</u>) mixture 50 μmol
oligonucleotide (RT-NTRNA7), 10 mM DTT and 2÷5÷mM dNTP

was added. After an incubation of 10 min at 70°C, 20
units of AMV reverse transcriptase (Boehringer, Mannheim,
Germany) and 40 units of RNase inhibitor (Boehringer,
Mannheim, Germany) were added. The RT reaction was done
5 at 42°C for 1 h. 1/3 of this reaction mixture was used
for the PCR reaction as described earlier.

17. Transfection and expression

HEKT cells were put in a 6 well plate at 2×10^5
10 cells/well and grown for 24 h. 2 µg pDNA with FuGene TM 6
Transfection reagent (Boehringer, Mannheim, Germany) was
added to the cells. 48 h after transfection the cells
were lysed in 100 µl PBS, pH 7.4 - 5 mM EDTA - 0.5%
Nonidet P40. The soluble fraction was isolated after 5
15 min centrifugation at 10,000 g. The pellet was
resuspended in 100 µl PBS, pH 7.4.

18. DNA vaccination

Plasmid DNA was used at a concentration of 1
20 µg/µl. Three intramuscular injections were given at three
weeks intervals. Serum was taken two weeks after every
immunization, pooled and analyzed in an ELISA for
antibody response towards the extracellular part of the
M2 protein (see Materials and Methods hereinabove).

25

19. ELISA II

Microtiterplates were coated with 1 µg/ml M2, expressed
in Sf9 insect cells (Black et al., 1993a, b). The
remainder of the procedure was as described in the
30 earlier section of Materials and Methods.

20. List of plasmids
20.1 E. coli

pATIPM2m1 : plasmid that contains the uninterrupted
35   m2 gene from A/PR/8/34
pIPM2hB2Mm2s2 : plasmid for the expression of
IPM2hB2Mm, with the correct amino terminus of M2

pPLcIPM2HBc : expression plasmid for IPM2HBc, with
four amino acids between the initiating methionine
and the amino terminus of M2e

pPLcIPM2HBcm : expression plasmid for IPM2HBcm, with
the correct amino terminus of M2e. Sequence of M2 is
5   derived from A/PR/8/34

pPLcIM2HBcm : expression plasmid for IM2HBcm, with
the correct amino terminus of the universal M2

10 20.2 <u>L. lactis</u>

pT1TT : plasmid for the expression of TTFC

pT1PM2LTT : expression of IPM2TT, with leucine
codons adapted for <u>L. lactis</u>. Sequence of M2e is
derived from A/PR/8/34

15   pT1PM2LTTIL2 : expression of IPM2TT, with adapted
leucine codons, in combination with mIL2

pT1PM2LTTIL6 : plasmid for the expression of IPM2TT,
with adapted leucine codons, in combination with
mIL6

20   pT1HBc : plasmid for the expression of HBc

pT1HBcIL2 : expression of HBc in combination with
mIL2

pT1HBcIL6 : expression of HBc in combination with
mIL6

25   pT1PM2HBc : plasmid for the expression of IPM2HBcm.
Sequence of M2e is derived from A/PR/8/34

pT1PM2HBcIL2 : expression of IPM2HBcm in combination
with mIL2

pT1PM2HBcIL6 : expression of IPM2HBcm in combination
30   with mIL6

pT1M2HBc : plasmid for the expression of IM2HBcm,
with the universal sequence for M2e

pT1M2HBcIL2 : expression of IM2HBcm in combination
with mIL2

35   pT1M2HBcIL6 : expression of IM2HBcm in combination
with mIL6

pT1PM2LHBc : plasmid for the expression of IPM2HBcm,
with leucine codons adapted for <u>L. lactis</u>

pT1PM2LHBcIL2 : expression of IPM2HBcm, with adapted leucine codons, in combination with mIL2

pT1PM2LHBcIL6 : plasmid for the expression of IPM2HBc, with adapted leucine codons, in combination with mIL6

pT1M2LHBc : expression of IM2HBcm, with leucine codons adapted for <u>L. lactis</u>

pT1M2LHBcIL2 : expression of IM2HBcm, with adapted leucine codons, in combination with mIL2

pT1M2LHBcIL6 : expression of IM2HBcm, with adapted leucine codons, in combination with mIL6

pT1cM2L : plasmid for the expression of the cytoplasmic form of M2e, with leucine codons adapted for <u>L. lactis</u>.

pT1cM2LC3d : expression of cM2LC3d, with adapted leucine codons

pT1cM2LC3d3 : expression of cM2LC3d3 (with three consecutive C3d domains), with adapted leucine codons

pT1sM2LX : plasmid for the expression of the secreted and anchored form of M2e, with leucine codons adapted for <u>L. lactis</u>

pT1sM2LC3d : expression of sM2LC3d, with adapted leucine codons

pT1sM2LC3d3 : expression of sM2LC3d3 (with three consecutive C3d domains), with adapted leucine codons

<u>20.3</u>

pUCM2   :   plasmid that contains the uninterrupted m2 gene from A/Ann Arbor/6/60

pCDNA3   :   basic vector for eukaryotic gene expression

pCIM2   :   plasmid used for DNA vaccinations, it carries the uninterrupted m2 gene from A/Ann Arbor/6/60

pCIM2HBcm :   plasmid used for DNA vaccinations, it carries <u>im2hbcm</u>

31

pCIP3M2HBcm: plasmid used for DNA vaccinations, it contains three times the extracellular domain of the M2 protein genetically fused to the Hepatitis B core protein. The fusion protein, IP3M2HBcm starts with the correct amino terminus of M2e. Sequence of M2 is derived from A/PR/8/34.

EXPERIMENTAL SECTION

1. Construction of pATIPM2m

The RNA segment 7 of the influenza A virus, A/PR/8/34 (H1N1), was cloned by a procedure as described for RNA segment 4 in Min Jou et al., 1980. The resulting plasmid was named pATIPMA and is commercially available (LMBP catalogue 1992, no. 1774).

The mRNA of the M2 protein is not a collinear transcript of RNA segment 7. Indeed, an intron of 689 nucleotides had to be removed (Lamb et al., 1981).

In the plasmid pATIPMA, StuI cuts after the first nucleotide of the second exon (see figure 1a). This nucleotide was included in the synthetic oligonucleotides, that were used to code for the first exon. The synthetic first exon, encoding the amino-terminus of the mature M2 protein, was designed to contain a single stranded GATC overhang at its 5' end. This allowed us to make the connection to a preceding BamHI site in the vector pATIPMA and to replace the original first exon.

Furthermore codon usage was optimized for expression in E. coli.

Next, we introduced, by site-directed mutagenesis (Stanssens et al., 1989), a BclI site at the junction between the extracellular part and the membrane anchoring region of the M2 protein (see figure 1 b). The amino acid sequence of the extracellular part was not changed. The resulting plasmid, pATIPM2m1, carries the uninterrupted m2 gene of A/PR/8/34.

2. Construction of IPM2hB2Mm

Parker and Wiley (1989) expressed human ß2-microglobulin in the periplasm of E. coli by making use of the plasmid p714. This plasmid contains the coding region for ß2-microglobulin preceded by the signal sequence of the outer membrane protein A of E. coli (OmpA-ss) (see figure 2a). The OmpA signal sequence is required for the translocation of the protein, to which this sequence is fused, to the periplasm. The signal sequence is cleaved off after transport. On plasmid p714, human ß2-microglobulin is under control of both the lipoprotein (lpp) and lacUV5 promoter. Addition of 1 mM IPTG to a mid-log phase culture leads to the production of ß2-microglobulin.

The coding sequence of the extracellular part of the M2 protein, isolated as a BamHI-BclI fragment from pATIPM2m1, was inserted between the signal sequence of ompA and the human ß2-microglobulin (for details see figure 2a). Due to the construction, there were 9 additional nucleotides between the end of the ompa signal sequence and the beginning of the m2 fragment, which had to be removed (see figure 2b). This was done by looping out mutagenesis according to Nakamaye and Eckstein, 1986. As a result, the plasmid pIPM2hB2Mm2s2 was obtained.

3. Localization of the IPM2hB2Mm

A freshly grown preculture of C3000 containing p714 or pIPM2hB2Mm2s2 was diluted 1/100 in LB with ampicillin. As described above, the hb2m and ipm2hb2mm genes are under control of the lacUV5 promoter. When the cultures reached a density of about 5.5×10^8 bacteria/ml, they were divided in two and one half of each culture was induced with 1 mM IPTG. After 3 h induction, the bacteria were harvested and fractionated. The periplasm of the bacteria was isolated by osmotic shock (Neu and Heppel, 1965). The remainder of the bacteria was sonicated (Vibra cell, Sonics & Materials Inc., Danbury, Conn., USA) and centrifuged for 10 min at 16000 g, to isolate the

cytoplasm. The different samples were analyzed on a SDS
15% PAGE-gel. Human B2M and the fusion protein IPM2hB2Mm
were transported to the periplasm, whereas the
precursors, still containing the signal sequence,
5 remained associated with the bacteria. Determination of
the amino-terminus of the mature IPM2hB2Mm (by courtesy
of Dr. J. Vandekerckhove) by automated Edman degradation
on a model 470A gas-phase sequencer coupled to a model
120A on-line phenylthiohydantoin amino acid analyzer
10 (Applied Biosystems, Foster City, CA., USA), demonstrated
that the OmpA signal sequence was correctly cleaved off.

4. Purification of IPM2hB2Mm

        The fusion protein IPM2hB2Mm could be expressed
15 efficiently in the periplasm of E.coli. Whereas
performing an osmotic shock is a critical procedure,
especially on large volumes, Steidler et al. (1994)
previously described an elegant system, based on the
controlled expression of the Kil protein, to release
20 periplasmic proteins in the growth medium.

        The kil gene is present on a compatible plasmid
under the tightly regulated P_L promoter, the leftward
promoter of phage λ (Remaut et al, 1981). The plasmid
pcI857K1 also carries the temperature sensitive repressor
25 of the P_L promoter, cI857. The fusion protein IPM2hB2Mm is
synthesized upon induction with 1 mM IPTG and at the end
of the production phase, the culture is switched from
28°C to 42°C to induce Kil.

        A fermentation (BioFlo IV fermentor, New
30 Brunswick Scientific Co., Edison, N.J., USA) was carried
out using the standard induction procedure described
above. The culture was centrifuged in a contifuge 17RS
(Heraeus Instruments, Hanau, Germany) at 11000 g and the
growth medium was isolated. The sodium chloride
35 concentration of the growth medium was adjusted to 300 mM
and buffered with 20 mM MES (2-(N-
morpholino)ethanesulphonic acid), pH 6.5. This solution
was loaded on a DEAE Sephacel column (ϕ = 5 cm, h = 6.5

cm), equilibrated with 20 mM MES, pH 6.5 - 300 mM NaCl. Under these conditions IPM2hB2Mm did not bind to the matrix. The ammonium sulphate concentration of the flow through was brought to 0.8 M with a 3.8 M $(NH_4)_2SO_4$

5   solution, pH 7. The mixture was loaded on a Phenyl Sepharose column (ϕ = 5 cm, h = 17 cm), equilibrated in 20 mM Tris-HCl, pH 7.5, 0.8 M $(NH_4)_2SO_4$. A decreasing ammonium sulphate concentration gradient starting from 0.8 M and going to 0, did not release the bound fusion

10   protein. This was achieved by eluting the column with a pH gradient from 20 mM Tris-HCl, pH 7.5 to 5 mM NaAc, pH 5.5. The peak fractions were pooled and diluted ten times in 20 mM diethylamine (DEA), pH 8.5.

    The complete mixture was loaded on a Sepharose

15   Q column (ϕ = 0.8 cm, h = 2.3 cm), equilibrated with 20 mM DEA, pH 8.5. The protein was eluted from the column with a salt gradient from 0 to 1 M. The peak fractions were pooled and loaded on a Sephacryl S-100 gel filtration column (ϕ = 1.5 cm, h 47 cm). Only one peak

20   with the expected molecular weight of about 15 kDa was observed. This purified IPM2hB2Mm was used to immunize mice for preparing hybridomas, secreting monoclonal antibodies directed against the M2 protein.

25   **5. Production of monoclonal antibodies to the M2 protein**
    Balb/c mice were immunized three times with 2.5 µg purified IPM2hB2Mm. For the first injection a complete dose of Ribi adjuvant was used. The second and third immunization were performed in the presence of

30   50 µg MPLA. The injections were given with an interval of three weeks. Three days after the last immunization, spleen cells were isolated and fused with myeloma cells SP2/0-AG14 using standard protocols (Köhler and Milstein, 1975). Supernatants from different immunoglobulin

35   producing cell clones were tested in ELISA and Western blot for reactivity against the other fusion protein IPM2HBcm (described further). The Hepatitis B core protein alone was used as a control to eliminate false

positive clones. The isotype of the antibody was determined (Isostrip, Boehringer, Mannheim, Germany). Two different immunoglobulin subtypes that recognized the extracellular part of the M2 protein were obtained, an
5 IgM and an IgG2a. Especially the IgG2a antibody was used in further experiments.

6. Expression of HBc and IPM2HBcm

Expression of proteins under control of the P_L
10 promoter was performed by shifting an exponentially growing culture from 28°C to 42°C (Remaut et al., 1981). A saturated preculture of MC1061 [pcI857] containing the plasmid pPLc245 (control), pPLcA1 (carrying the hbc gene) or pPLcIPM2HBcm (containing the fusion gene ipm2hbc)
15 respectively, was diluted 1/100 in LB medium (50 μg/ml kanamycin and 100 μg/ml ampicillin) and grown for about 4 h at 28°C under shaking. When the cultures reached a density of 4.5×10^8 to 5.5×10^8 bacteria/ml, they were split, one half was incubated for 4 h at 28°C, the other
20 half was switched to 42°C. The bacteria were concentrated by centrifugation (2 min at 16000 g in a microcentrifuge).

The culture medium was removed and the bacteria were resuspended in TE buffer (10 mM Tris-HCl - 1 mM
25 EDTA, pH 7.6). The bacteria were opened by sonication (Vibra cell, Sonics & Materials Inc., Danbury, Conn., USA) and the bacterial debris were pelleted for 10 min at 16000 g in a microcentrifuge. The supernatant was isolated and the pellet was resuspended in TE buffer. The
30 samples were analyzed on a SDS 12.5% PAGE-gel, in a Western blot and on a dot blot.

7. Large scale production of IPM2HBcm

The strain MC1061 [pcI857, pPLcIPM2HBcm] was
35 grown in a BioFlo IV fermentor (New Brunswick Scientific Co., Edison, N.J., USA). When the culture reached a density of about 5.5×10^8 cells/ml, the temperature was increased to 42°C. After three hours of induction, the

culture was centrifuged in a contifuge 17RS (Heraeus Instruments, Hanau, Germany) at 11,000 g. The bacteria were collected and resuspended in a volume (in ml) buffer (50 mM Tris-HCl pH 8 - 150 mM NaCl - 5% glycerol with one
5 protease inhibitor cocktail tablet (Complete™; Boehringer, Mannheim, Germany) per 25 ml) corresponding to two times the weight (in g) of the pelleted bacteria. This suspension was treated with 1 mg/ml lysozyme (freshly dissolved in 25 mM Tris-HCl, pH 8) for half an
10 hour on ice. Subsequently, the bacteria were lysed with 0.2% Triton X-100 in the presence of 25 mM EDTA, pH 8. After 30 min incubation on ice, the lysates were centrifuged for 1 h in a Sorvall SS-34 rotor (Du Pont Company, Wilmington, DE, USA) at 48000 g. The supernatant
15 was removed and used for purification of IPM2HBcm.

8. Immunization with IPM2HBcm

Balb/c mice were injected three times intraperitoneally with purified IPM2HBcm in the presence
20 of adjuvant. Control mice received only PBS buffer, pH 7.4 and adjuvant. For the first immunization half a dose of Ribi adjuvant was used. In the second and third injection we used 25 μg MPLA and 25 μg MDP.

Mice were immunized intranasally three times by
25 applying a light ether anaesthesia, after which 50 microliter antigen solution in PBS buffer (containing either 10 microgram IPM2HBcm or IM2HBcm without any adjuvant) is put in the nostril.

30 9. Expression in L. lactis

Single colonies from L. lactis strain MG 1363, containing the plasmid pT1HBc, pT1PM2HBc or pT1M2HBc, respectively, or the derivatives with mIL2 (pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2) or mIL6 (pT1HBcIL6,
35 pT1PM2HBcIL6 and pT1M2HBcIL6), were inoculated in 10 ml GM17E each. MG1363 [pTREX1] was used as control. The bacteria were grown for about 16 h at 28°C. The cells were collected by centrifugation at 2000 g for 20 min

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(Sorvall 11 RT6000 D). The growth medium was isolated and
the bacteria were resuspended in 250 μl TE. Following
resuspension, an additional 250 μl TE supplemented with
10 mg/ml lysozyme and 200 u/ml mutanolysin was added.
5 This mixture was incubated for 10 min at 37°C and then
put on ice for 5 min. Then 500 μl Laemmli sample buffer
(100 mM Tris-HCl pH 6.8 - 5% SDS - 1.2M ß-mercaptoethanol
- 0.008% bromophenol blue - 16% glycerol) was added and
the samples were boiled for 5 min. An equivalent of 1 ml
10 original culture volume, or 10^9 bacteria was analyzed on a
SDS 12.5% PAGE-gel. The production of mIL2 or mIL6 in the
culture supernatant was evaluated in a bio-assay based on
the proliferation of CTLL2-cells (mIL2, Gillis et al.,
1978) or the proliferation of a B-cell hybridoma, 7TD1
15 (mIL6, Van Snick et al., 1986).

10. Passive immunization

        The purified preparation of IM2HBcm particles
was used to immunize 7 weeks old female Balb/c mice. A
20 total of 40 mice were immunized with 10 pg IM2HBcm. A
control group of 40 mice only received buffer. A total of
three injections combined with appropriate adjuvant were
given at three weeks intervals (see Materials and
Methods). Two weeks after the third immunization 28 mice
25 from each group were bled and serum was isolated (see
Materials and Methods). This serum was administered
intraperitoneally to naive mice 24 h before infection.
This process is called passive immunization. Twelve mice
received 800 μl serum from IM2HBcm immunized mice and
30 another 12 mice received serum from the control group.
These 24 mice and the remaining 24 immunized mice were
challenged with 5 LD_{50} m.a. X47 three weeks after the
third immunization. The virus was administered
intranasally in a total volume of 50 μl after ether
35 anaesthesia. Morbidity was followed by measuring rectal
temperature and weight every other day.

11. Constructs for DNA vaccination (Fig. 29)

The mammalian expression vector, pCDNA3 (Invitrogen, Leek, The Netherlands), which carries the cytomegalovirus promoter was used to make the different
5 DNA vaccination vectors.

The uninterrupted m2 gene was isolated by RT-PCR from the influenza A virus A/Ann Arbor/6/60 (see Materials and Methods). The amplified fragment was cut with BglII and XbaI and inserted into the BglII and XbaI
10 opened pUC19. This plasmid was called pUCM2. The sequence of the m2 gene was determined and shown to correspond to the cold adapted form of the gene. The m2 gene was isolated from pUCM2 as a 321 bp EcoRI-XbaI fragment and inserted into the EcoRI and XbaI opened pCDNA3. This
15 resulted in plasmid pCIM2.

Two fusion genes, ip3m2hbcm and im2hbcm, were also inserted into pCDNA3. The im2hbcm gene was amplified by PCR from pPLcIM2HBcm. This fragment was cut with SpeI and phosphorylated with T4 polynucleotide kinase. This
20 630 bp fragment was inserted in the EcoRV and XbaI opened pCDNA3. The resulting plasmid was called pCIM2HBcm.

During the construction of pPLcIPM2HBc (see figure 3a) plasmids were also obtained with two and three inserted M2e fragments. These plasmids were called
25 pPLcIP2M2HBc and pPLcIP3M2HBc, respectively. The ip3m2hbcm gene was amplified by PCR from pPLcIP3M2HBc. This fragment was cut with SpeI, phosphorylated with T4 polynucleotide kinase and inserted in the EcoRV and XbaI opened pCDNA3. This plasmid was called pCIP3M2HBcm.
30 Plasmid DNA was isolated with an EndoFree Plasmid Giga kit (Qiagen, Hilden, Germany). The concentration pDNA was determined by spectrophotometric analysis.

35 12. Expression in HEKT cells

The plasmids pCDNA3, pCIM2, pCIM2HBcm and pCIP3M2HBcm were transfected to HEKT cells (see Materials

and Methods). 48h after transfection the cells were lysed
and analyzed in a Western blotting experiment.

13. Analysis of the serum

5   Two weeks after every immunization serum
samples were taken and analyzed in an ELISA. In panel A
from figure 31 the two vectors, which can express the HBc
fusion proteins are compared with the control vector. The
ELISA was performed as described in Materials and Method.

10

RESULTS

1. Construction of IPM2HBcm

           The plasmid pPLcAl (see figure 3a) contains the
hepatitis b core (hbc) gene under control of the P_L
15 promoter of bacteriophage λ (a gift from Dr. Nassal). The
346 bp NcoI-XbaI HBc fragment, isolated from pPLcAl, was
inserted into the NcoI and XbaI opened pMa581, a
derivative of pMa58. This plasmid was called pMaHBc. At
the 5' end of the hepatitis B core, directly following
20 the start codon, we introduced a BamHI site by site-
directed mutagenesis (Stanssens et al., 1989), correctly
positioned in the reading frame of HBc (for details see
figure 3a and b). The resulting plasmid was named
pMaHBcm. The information coding for the extracellular
25 part of the M2 protein was cloned as a 72 bp BamHI-BclI
fragment, derived from pATIPM2m1, into the BamHI opened
pMaHBcm, resulting in the vector pIPM2HBc. The hbc gene
in the expression vector pPLcAl was then replaced by the
418 bp NcoI-XbaI m2hbc fragment, creating pPLcIPM2HBc.
30 Due to the construction, four amino acids extra were
present between the first methionine and the start of the
extracellular part of the M2 protein and had to be
removed (see figure 3c). This was done by looping out
mutagenesis (Deng and Nickolov, 1992). The resulting
35 plasmid was named pPLcIPM2HBcm (see figure 3a and c).

2. Expression of the fusion protein

The plasmids pPLc245 (control), pPLcA1 (hbc gene) and pPLcIPM2HBcm (ipm2hbc gene) were transformed to MC1061 [pcI857]. After culture and induction, the
5 bacteria were lysed by sonication. The lysates were centrifuged and an aliquot of the supernatants was loaded on a SDS 12.5% PAGE-gel (see figure 4). The same fractions were also analyzed by a Western blot. Two different monoclonal antibodies were used : an antibody
10 specific for the Hepatitis B core protein and a monoclonal antibody (IgG2a) directed against the extracellular part of the M2 protein.

The monoclonal antibody against Hepatitis B core revealed two different bands (see figure 5A), one
15 corresponding to the Hepatitis B core protein and the other to the fusion protein. The latter protein has a lower mobility, corresponding to the insertion of the extracellular domain of the M2 protein. The presence of the M2 fragment was confirmed by using the antibody
20 specific for the extracellular part of the M2 protein (see figure 5B).

The N-terminal amino acid sequence of IPM2HBcm was determined (Dr. J. Vandekerckhove) by automated Edman degradation on a model 470A gas-phase sequencer coupled
25 to a model 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Foster City, CA., USA). This analysis revealed the N-terminal sequence Ser-Leu-Leu, which is exactly the same as the amino terminal sequence of the M2 protein of the influenza A virus
30 (figure 6). The first amino acid, methionine, was removed in E. coli. The amino-terminus of the fusion protein thus corresponds to that of the wild type M2 protein (table 1; Lamb et al., 1985).

Hepatitis B core, also when expressed in E.
35 coli, spontaneously associates to form particles, indistinguishable from the viral core particles circulating in the blood of Hepatitis B infected patients (Cohen and Richmond, 1982). Clarke and co-workers (1987)

showed that a peptide inserted at the amino terminus of the Hepatitis B core protein could be detected at the surface of the particle.

Electron micrographs (Dr. G. Engler) showed
5 that the IPM2HBcm fusion protein was able to form similar particles. To investigate whether the insertion of the extracellular part of the M2 protein resulted in the surface localization of this fragment, soluble fractions, containing HBc or IPM2HBcm, were loaded on a
10 nitrocellulose membrane in a dot blot. The dot blots were treated with a monoclonal antibody directed against HBc or against M2. Figure 7 clearly shows a signal in the soluble pPLcIPM2HBcm fraction, when revealed with the antibody directed against the M2 protein (panel B). Since
15 the soluble fraction is loaded in a native state onto the nitrocellulose membrane, we conclude that the epitope is located at the surface of the Hepatitis B core particle.

3. Purification of IPM2HBcm

20   The bacterial lysates were prepared as described in Materials and Methods. The concentration of Tris-HCl, pH 8 and NaCl were adjusted to 20 mM and 50 mM respectively. This mixture was loaded on a DEAE Sepharose column (ϕ = 2.5 cm, h = 5.5 cm), equilibrated with 20 mM
25 Tris-HCl, pH 8-50 mM NaCl. The fusion protein was not retained on the column. To the flow through 3.8 M $(NH_4)_2SO_4$, pH 7, was added to a final concentration of 1.2 M. This mixture was incubated under stirring in the cold room during 16h. The precipitate was removed over a CF11
30 cellulose column (ϕ = 2.5 cm, h = 3.5 cm). The column was eluted with PBS, pH 7.4. The eluate of about 50 ml was concentrated in a Centiprep 30 (Amicon Corporation, Danvers, Ill., USA) to 5 ml and loaded on a Sephacryl S-300 column (ϕ = 2.5 cm, h = 91 cm), which was
35 equilibrated with PBS, pH 7.4. The peak fractions were pooled and the concentration of IPM2HBcm was determined in an ELISA, The LPS content was assayed (LAL Coatest® Endotoxin purchased from Endosafe Inc., Charleston, SC.,

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USA) and was sufficiently low (5 to 9 ng/50 µg IPM2HBcm)
not to interfere with immunization.

4. Immunization

5   The purified preparation of IPM2HBcm particles
was used to immunize 7 weeks old female Balb/c mice. Four
different groups of 12 mice were evaluated. The first
group received 50 µg IPM2HBcm, the second 10 µg, the
third 5 µg and the fourth a control group, only received
10 buffer with adjuvant. A total of three injections were
given with the appropriate adjuvant. The injections were
administered with three weeks interval. Three weeks after
the last inoculation, the mice were challenged with 5 LD_{50}
m.a. A/PR/8/34. The virus was administered intranasally
15 in a total volume of 50 µl after ether anaesthesia.
Morbidity was followed by measuring rectal temperature
(figure 8 A1) and weight (figure 8 A2) every other day.
          All mice immunized with IPM2HBcm showed a
significant degree of protection against the following
20 influenza challenge. Depending on the administered dose,
9 to 11 mice out of 12 survived the influenza infection,
versus only 2 out of 11 for the control group (see figure
8B).

25 5. Analysis of the serum samples
          One day prior to the first (bleeding a) and two
weeks after every injection (bleeding b, c and d) blood
samples were taken. Three weeks after the challenge, when
the mice had recovered sufficiently from the influenza
30 infection, a last blood sample (e) was taken. The serum
was analyzed in an ELISA (see Materials and methods) to
identify IgG antibodies directed towards the
extracellular part of the M2 protein. To do so, we made
use of the other fusion protein, IPM2hB2Mm. One half of
35 the microtiter plate was coated with human ß2-
microglobulin, the other half was coated with the fusion
protein IPM2hB2Mm, both as unpurified culture
supernatant. The concentration of IPM2hB2Mm used was 1

μg/ml. The same concentration of total protein was used
in both set ups. Therefore, the hB2M content of the
culture supernatant of bacteria expressing hB2M had to be
adjusted to 1 μg/ml by adding purified hB2M (Sigma

5 Chemical Co., St. Louis, Mo., USA). Dilution series (1/3)
of the different serum samples, starting from 1/50, were
loaded on the hB2M and IPM2hB2Mm, coated wells. The ELISA
was further developed as described in Materials and
methods.

10   To obtain the value for the specific reactivity
towards the extracellular part of the M2 protein, the
absorbance of hB2M at a given dilution was subtracted
from the absorbance of IPM2hB2Mm of the corresponding
dilution. Figure 9 clearly demonstrates a high antibody

15 response to the extracellular part of the M2 protein, in
the mice which received three injections with the
vaccine. The titer in the serum was further increased
after the challenge.

20 6. Construction of IM2HBcm

        It is the aim of the present invention to make
a universal vaccine against influenza A viruses. In the
vaccination studies described above, we showed protection
against the influenza virus from which the original M2

25 sequence was derived, A/PR/8/34 (homologous protection).
The extracellular part of the M2 protein from this virus
differs from most other viruses sequenced to date, by
only one amino acid (see table 1). Therefore, a construct
was made in which the glycine at position 20 was changed

30 to aspartic acid.

        To do so we made use of an intermediate vector
in the construction of pPLcIPM2HBcm, pMaIPM2HBc2 (see
figure 3a). The plasmid pMaIPM2HBc2 does not yet contain
the mutated m2 (deletion of 12 extra nucleotides)

35 fragment, which starts at the first mature codon of the
M2 protein. Therefore this fragment was isolated from
pPLcIPM2HBcm by cutting with SgrAI and EcoRI. This 499 bp
SgrAI-EcoRI fragment was cloned into the SgrAI and EcoRI

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opened vector pMaIPM2HBc2, which resulted in the
construction of pMaIPM2HBc3 (see figure 10).

By site-directed mutagenesis according to Deng
and Nickoloff (1992) the sequence of the extracellular
5 part of the M2 protein was changed to the more universal
M2 sequence (Gly20 -> Asp). The new plasmid was called
pIM2HBcm. The sequence was determined on a model 373A
sequencer (Applied Biosystems, Foster city, CA., USA) and
shown to contain the desired mutation. The mutated M2
10 fragment was isolated from pIM2HBcm as a 499 bp SgrAI-
EcoRI fragment and reintroduced into the expression
vector pPLcIPM2HBcm, opened with SgrAI and EcoRI, to
create pPLcIM2HBcm.

15 7. Expression of IM2HBcm

Strain MC1061 [pcI857] containing respectively
pPLc245, pPLcA1, pPLcIPM2HBcm or pPLcIM2HBcm was cultured
as described in the Experimental Section. The bacteria
were collected and opened by sonication. The soluble
20 fraction was isolated and the concentration of Hepatitis
B core protein or the derived fusion proteins was
determined in an ELISA. A soluble fraction containing 5
μg HBc or I(P)M2HBcm was analyzed on a SDS 12.5% PAGE-gel
(see figure 11). The same fractions were also analyzed in
25 a Western blot (see figure 12). The proteins of interest
were detected with an antibody directed against the
Hepatitis B core protein or with the monoclonal antibody
specific for the extracellular part of the M2 protein. It
can be concluded that the new fusion protein, IM2HBcm, is
30 expressed as efficiently as IPM2HBcm. Moreover the amino
acid change in the extracellular part of the M2 protein
(Gly20 --> Asp) has no effect on the binding of the
monoclonal anti-M2 antibody.

35 8. Immunization against heterologous challenge

A similar procedure as described in point 4 was
used to test the efficiency of IPM2HBcm and IM2HBcm to
protect mice versus heterologous challenge with

influenza. 10 microgram of IPM2HBcm or IM2HBcm (purified
in an identical way as IPM2HBcm) was used for
immunization. The mice were challenged with 30 HAU X-47.

All mice immunized showed a significant degree
5 of protection against the heterologous challenge. 8 (in
case of IPM2HBcm, p<0.05) or 12 (in case of IM2HBcm,
p<0.0001) mice out of 12 survived the influenza
infection, versus only 2 out of 11 in the control group
(figure 8C).

10   To test the effect of intranasal
administration, the same procedure was followed, but
instead of the intraperitoneal injection, the antigen was
administered intranasally. Also in this case, the
protection is evident: 12 (in case of IPM2HBcm, p<0.0001)
15 or 11 (in case of IM2HBcm, p<0.001) mice out of 12
survived the influenza infection, versus 2 out of 11 in
the control group (figure 8D).

9. Construction of vectors for the expression of M2-HBc
20 fusion proteins in L. lactis

The plasmid pTREX1 (Wells and Schofield, 1996)
was used to express the Hepatitis B core protein and two
M2-HBc fusion proteins, IPM2HBcm and IM2HBcm, in
Lactococcus lactis. This plasmid has a constitutive L.
25 lactis chromosomal promoter, P1, which is followed by the
translation initiation region of the E. coli
bacteriophage T7 gene 10 (Wells and Schofield, 1996). The
transcription terminator is derived from T7 RNA
polymerase. The plasmid pTREX1 also carries two genes for
30 resistance to erythromycin.

The expression plasmid, pTREX1, was cut with
SphI, leaving a 3'CATG extension which was removed with
Klenow DNA polymerase. The removed nucleotides were
included in the sense linker for PCR amplification of the
35 different genes. The linearized vector was then cut with
BamHI and treated with CIP (calf intestine phosphatase,
Boehringer, Mannheim, Germany).

The genes hbc, ipm2hbc and im2hbc were
amplified by PCR (see Materials and methods). The anti-
sense linker (HBca) was identical in all amplifications
and provided a SpeI and a BclI site after the stop codon
5 (see figure 13). For the amplification of ipm2hbc and
im2hbc the same sense oligonucleotide (M2s) could be
used, since the mutation Gly → Asp in the extracellular
part of the M2 protein is located further downstream.

The amplification of hbc from pPLcA1 was only
10 possible after the vector had been linearized with ScaI.
The amplification reaction that produced a sufficient
amount of fragment, under the most stringent conditions,
was used for further cloning. The amplified fragment,
hbc, ipm2hbc or im2hbc, was cut with BclI, phosphorylated
15 with T4 polynucleotide kinase and inserted in the SphI
and BamHI opened pTREX1 (see figure 14). The new plasmids
were called pT1HBc, pT1PM2HBc (in which the extracellular
part of the M2 protein is derived from the virus
A/PR/8/34) and pT1M2HBc (in which the sequence of the
20 extracellular part of the M2 protein corresponds to the
type present in nearly all human influenza A viruses
sequenced to date), respectively. The sequence of the
inserted fragment was determined on a model 373A
sequencer (Applied Biosystems, Foster City, CA., USA) and
25 shown to be correct.

In view of using Lactococcus lactis as an
improved vaccine delivery vehicle, two murine cytokines,
interleukin 2 (mIL2) and interleukin 6 (mIL6) were
inserted as second cistrons in the same operon as the
30 antigen. In that way we could obtain bacteria expressing
the antigen, e.g. IM2HBcm, together with secreted murine
interleukin 2 or 6. To obtain secretion of the
interleukins into the growth medium, they were fused in
frame to the lactococcal usp45 secretion signal peptide
35 (van Asseldonk et al., 1990). The plasmids pT1HBc,
pT1PM2HBc and pT1M2HBc were cut with SpeI and treated
with CIP. The murine interleukin 2 gene was isolated as a
572 bp XbaI-SpeI fragment from plasmid pL2MIL2 (Steidler

et al., 1995). This fragment was inserted into the SpeI opened pT1HBc, pT1PM2HBc and pT1M2HBc giving rise to pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2, respectively. In an analogous way the murine interleukin 6 gene was

5 isolated as a 687 bp XbaI-SpeI fragment from pL2MIL6 (Steidler et al., 1996) and inserted into the SpeI opened vectors, pT1HBc, pT1PM2HBc and pT1M2HBc, to create pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6, respectively.

10 10. Expression of HBc and M2HBc in L. lactis

Lactoccoccus lactis strain MG1363 (Gasson, 1983) containing the plasmids for the expression of the antigen alone (pT1HBc, pT1PM2HBc and pT1M2HBc) or in combination with mouse interleukin 2 (pT1HBcIL2,

15 pT1PM2HBcIL2 and pT1M2HBcIL2) or mouse interleukin 6 (pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6) were cultured as described in Materials and Methods. MG1363 [pTREX1] was used as control.

An equivalent of 10^9 bacteria was analyzed by

20 SDS 12.5% PAGE. The expression of the Hepatitis B core and the M2-HBc fusion proteins were analyzed by Western immunoblotting (see figure 15) carried out as described in Materials and methods. The expression of IM2HBc in MG1363 [pT1M2HBcIL6] was not as high as in the other

25 constructs. By screening different colonies a clone could be isolated with comparable expression levels.

The production and secretion of interleukins into the growth medium was analyzed in a biological assay. The biological activity of mIL2 was assayed by the

30 proliferation of a T-cell line, CTLL2 (Gillis et al., 1978) as compared to a human IL2 standard. The biological activity of mIL6 was measured by the proliferation of a B-cell hybridoma, 7TD1 (Van Snick et al., 1986). Table 2 gives an overview of the level of interleukin 2 and 6 per

35 ml culture medium produced by the different expression plasmids. The supernatant of cultures producing mIL6 did not lead to proliferation in a mIL2 assay and vice versa.

Table 2

Plasmid	mIL2 production	mIL6 production
pT1HBcIL2	410 ng/ml	-
pT1PM2HBcIL2	481 ng/ml	-
pT1M2HBcIL2	359 ng/ml	-
pT1HBcIL6	-	1020 ng/ml
pT1PM2HBcIL6	-	772 ng/ml
pT1M2HBcIL6	-	802 ng/ml

11. Adaptation of the coding sequence of M2e to
expression in L. lactis
         Since the two fusion proteins, IPM2HBcm and
IM2HBcm could hardly be detected in a Western blot, we
proceeded to augment the production of these two fusion
proteins by adapting the codon usage of the extracellular
part of the M2 protein to L. lactis (van de Guchte et
al., 1992).
         At the 5' end of the extracellular part of the
M2 protein we observed two consecutive leucine codons
(CUG CUG) that were optimal for expression in E coli
(68%), but poor for translation in L. lactis (8%,
percentages described in van de Guchte et al., 1992).
Therefore these codons were changed to UUA. The genes for
ipm2hbc and im2hbc were amplified by PCR from
respectively pPLcIPM2HBcm or pPLcIM2HBcm, with a new
sense primer, M2Ls, containing the two changed leucine
codons (see figure 13). As anti-sense primer we used
again HBca (see figure 13). The cloning of the genes was
analogous as depicted in figure 14. The vectors so
created were called pT1PM2LHBc and pT1M2LHBc.
         The expression level of the mutated M2HBc
proteins, compared to the original fusion proteins, was
analyzed in a Western blot (see figure 16). The
expression level of the M2HBc fusion proteins with the L.
lactis adapted leucine codons, was indeed much higher. It

is concluded that the adaptation of codon usage to the L. lactis translation machinery, has a positive effect on the level of protein produced. In a similar way as described above, the murine interleukin 6 gene was

5 inserted into pT1PM2LHBc and pT1M2LHBc, giving rise to pT1PM2LHBcIL6 and pT1M2LHBcIL6, respectively.

12. Construction of M2C3d in Lactococcus lactis

A second carrier protein, C3d, is also an

10 attractive molecule for the presentation of the extracellular part of the M2 protein. Dempsey et al. (1996) demonstrated that the attachment of an antigen to three consecutive C3d molecules, was much more efficient in producing a high antibody response than the antigen

15 administered in complete Freund's adjuvant.

The universal sequence of the extracellular part of the M2 protein, with the adapted leucine codons, was used for making a fusion to the amino-terminus of the first C3d molecule. The coding sequence for three

20 different fusion proteins were constructed. In the first example the M2C3d3 fusion protein is expressed in the cytoplasm of L. lactis (cM2C3d3), similar to the M2HBc fusion proteins. In the second case the M2C3d3 protein is secreted into the growth medium by making an in frame

25 fusion to the usp45-signal sequence (sM2C3d3), and the last construct, which is a derivative of the secreted form, contains in addition an anchor sequence (spaX) after the last C3d molecule to attach the fusion protein covalently in the cell wall (sM2C3d3X).

30 The amplified C3d3 fragment was first subcloned in a derivative of pUC18, namely pUCB/S. pUC18 was linearized with HindII and a BglII linker was inserted. The resulting plasmid was then opened with SmaI and a SpeI linker was inserted, resulting in the plasmid pUCB/S

35 (see figure 18). Three succeeding copies of C3d were amplified from pSG5.C3d3.YL (a gift from Dr. D. Fearon) by PCR with the oligonucleotides C3ds and C3da (see figure 17). This amplified fragment was cut with BglII

and SpeI. The resulting 2830 bp BglII-SpeI fragment was cloned into the BglII and SpeI opened vector pUCB/S (see figure 18). The genes cm2 and sm2 were amplified by PCR. For the amplification of cm2 we used the sense

5 oligonucleotide M2Ls (see figure 13) and the anti-sense linker M2Ca, which carried for our purposes a BamHI site in the correct reading frame (see figure 17). The same anti-sense linker was used for the amplification of sm2. The sense oligonucleotide for the amplification of sm2,

10 M2LSs, started at the first codon of the mature M2 protein.

For the synthesis of the cytoplasmic form of M2C3d3, the information coding for the extracellular part of the M2 protein was inserted into pTREX1 analogous as

15 the m2hbc gene described above (see also figure 18). The amplified cm2 fragment was cut with BamHI (77 bp), phosphorylated with T4 polynucleotide kinase and inserted in the SphI and BamHI opened pTREX1, creating pT1cM2L.

For the synthesis of the secreted and anchored form of

20 M2C3d3, the information coding for the extracellular part of the M2 protein was inserted into pT1NX. The vector pT1NX carries the usp45-signal sequence (usp45-ss) and the anchor sequence derived from Staphylococcus aureus protein A (spaX). The plasmid pT1NX was cut with NaeI,

25 correctly positioned at the end of the usp45-ss and BamHI. The amplified fragment, sm2, was cut with BamHI and phosphorylated with T4 polynucleotide kinase. This 73 bp sm2 fragment was inserted into the NaeI and BamHI opened pT1NX, resulting in the plasmid pT1sM2LX (see

30 figure 18). One single C3d fragment, isolated from pUCC3d, can then be inserted into the BamHI site at the end of the cm2 or sm2 sequence. Afterwards one or two additional C3d copies can be inserted.

35 13. Construction of M2TTFC in Lactococcus lactis

A third carrier protein, tetanus toxin fragment C (TTFC), can also be used. TTFC has already been expressed in L. lactis under control of the P1 promoter,

51

pT1TT (Wells and Schofield, 1996). L. lactis expressing
TTFC in combination with mIL2 or mIL6 to raise the
antibody production, was successfully used in
immunization experiments (Patent GB 9521568.7).
5   As positive control for analysis of antibody response in
the present immunization experiments with L. lactis
expressing I(P)M2HBcm, a fusion was made between the
extracellular part of the M2 protein and the amino
terminus of TTFC.
10   The ttfc gene was amplified by PCR (see
Materials and methods) from pT1TT. The sense
oligonucleotide (TTFCs) provided a BamHI site, positioned
in the correct reading frame, before the second codon of
ttfc, corresponding to threonine. The anti-sense linker
15 (TTFCa) provided a SpeI and a BamHI site after the stop
codon (see figure 19). The amplification reaction that
produced a sufficient amount of fragment, under the most
stringent conditions, was used for further cloning (see
Materials and methods). The amplified ttfc fragment was
20 cut with BamHI, phosphorylated with T4 polynucleotide
kinase and inserted in the BclI opened pATIPM2m1 (see
figure 20). This plasmid construct was called pATIPM2TT.
From this plasmid the m2ttfc gene was amplified by PCR
(see Materials and methods) with M2Ls and TTFCa (see
25 figure 19). The amplified m2ttfc fragment was cut with
BamHI, phosphorylated with T4 polynucleotide kinase and
inserted in the SphI and BamHI opened pTREX1 (see figure
20). The new plasmid was called, pT1PM2LTT. In this
construct the extracellular part of the M2 protein is
30 derived from the virus A/PR/8/34, with the two leucine
codons adapted for use in L. lactis. The sequence of the
inserted fragment was determined on a model 373A
sequencer (Applied Biosystems, Foster City, CA., USA) and
shown to be correct.
35   The murine interleukin genes, mIL2 and mIL6,
were inserted in the same operon as m2ttfc. The murine
interleukin 2 gene was isolated as a 572 bp XbaI-SpeI
fragment from plasmid pL2MIL2 (Steidler et al., 1995).

52

This fragment was inserted into the SpeI opened pT1PM2LTT
giving rise to pT1PM2LTTIL2 (see figure 20). In an
analogous way the murine interleukin 6 gene was isolated
as a 687 bp XbaI-SpeI fragment from pL2MIL6 (Steidler et
5 al., 1996) and inserted into the SpeI opened vector
pT1PM2LTT to create pT1PM2LTTIL6 (see figure 20).

14. Expression of TTFC and M2TTFC in L. lactis
         Lactoccoccus lactis strain MG1363 (Gasson,
10 1983) containing the plasmids for the expression of the
antigen alone (pT1PM2LTT) or in combination with mouse
interleukin 2 (pT1PM2LTTIL2) or mouse interleukin 6
(pT1PM2LTTIL6) were cultured as described in Materials
and Methods. MG1363 [pT1TT] was used as a control.
15 An equivalent of 10^9 bacteria was analyzed by SDS 10%
PAGE. The expression of the IPM2TTFC fusion protein was
analyzed by Western immunoblotting (see figure 21)
carried out as described in Materials and Methods.
The production and secretion of interleukins into the
20 growth medium was analyzed by a biological assay. L.
lactis [pT1PM2LTTIL2] produced about 500 ng/ml mIL2 and
L. lactis [pT1PM2LTTIL6] about 1 μg/ml mIL6. These
results are comparable with the expression levels
obtained with I(P)M2HBcm in combination with the two
25 interleukins.

15. Construction of pACsgpM2C3d3 and generation of the
corresponding recombinant baculovirus
         The amplified sequence of the baculovirus gp67
30 secretion signal was cut with SpeI and HindIII, and then
subcloned in the SpeI-HindIII vector fragment of pUCC3d,
resulting in pUCsgp. After HindIII and NaeI digestion of
pUCsgp, the gp67 secretion signal was ligated with a
HindIII treated M2e fragment (universal sequence)
35 obtained from a PCR amplification (primers M2Ss and
UM2ECa). This construct, referred to as pUCsgpM2, was
digested with BamHI and subsequently recirculized by

53

ligation with the BglII-BamHI pUCC3d3 fragment containing
3 consecutive C3d fragments, yielding pUCsgpM2C3d3.

The latter fragment was excised after ligation
of the BamHI (dephosphorylated)-EcoRI pUCC3d fragment,
5 the BglII (desphosphorylated)-EcoRI pUCC3d fragment and
the BglII-BamHI pUCC3d fragment. The SpeI fragment of
pUCsgpM2C3d3 containing the sgpM2C3d3 fusion sequence was
then inserted behind the polyhedrin promoter by
exchangement with the SpeI-XbaI fragment of the
10 baculovirus transfer vector pACGP67A. The resulting
transfer vector, called pACsgpM2C3d3, was then used to
generate recombinant AcNPV/sgpM2C3d3 baculovirus by
calcium phosphate cotransfection of Sf9 insect cells with
BaculoGold baculovirus DNA (Pharmingen, San Diego, CA,
15 USA), following the procedure as described in King and
Possee (1992). The presence of the sgpM2C3d3 fusion
sequence behind the polyhedrin promoter in the genome of
the corresponding recombinant AcNPV/sgpM2C3d3 baculovirus
was confirmed by PCR analysis.
20
16. Expression of secreted M2C3d3 by Sf9 insect cells
Log-phase Sf9 insect cells were inoculated with
recombinant AcNPV/sgpM2C3d3 baculovirus at high
multiplicity of infection (> 10). Cells were subsequently
25 transferred to serum-free TC100 medium and further
incubated for 48 h before harvesting the supernatant.
Proteins were precipitated by adding an equal volume of
acetone (preequilibrated at -20°C) and subsequently
analyzed by Western blotting.
30   In a preferred construction, three or more
copies of the C3d protein are preceded by the
extracellular domain of the M2 protein.

17. Passive immunisation
35   The survival is shown in figure 28. In both
control groups only one mouse out of 12 survived the
lethal influenza challenge, while 11 out of 12 mice
immunized with 3 x 10 pg IM2HBcm or all passively

immunized mice were protected. This experiment
demonstrates that anti-M2 antibodies produced during the
vaccination account for the observed protection.

5   18. DNA vaccination

Table 3 shows the results of a DNA vaccination
experiment in which 12 mice injected with 3 x 100 μg
pCIM2 were compared with a control group injected three
times with 100 μg pCDNA3 for the survival against a

10 lethal challenge (5 LD_{50}) with m.a. X47. A partial
protection against a heterologous (immunising antigen =
universal M2, challenge = A/PR/8/34 derived M2) influenza
challenge could be demonstrated.

15 Table 3

vector	surviving mice/total number
pCDNA3 (control)	1/12
pCIM2 (complete m2 gene)	7/12

20

19. Expression in HEKT cells

The expression level of the complete M2 protein
is too low to be detected, in the soluble fraction and in
the pellet (see figure 30). It is possible that the

25 expression is kept low due to the ion channel activity of
the M2 protein, which can be toxic for the HEKT cells.
The two fusion proteins, IM2HBcm and IP3M2HBcm however
are well expressed. This experiment demonstrates that the
vectors used in the DNA vaccination studies can express

30 the protein, except maybe for pCIM2.

20. Analysis of the serum

A specific antibody response directed towards
the extracellular part of the M2 protein could be

35 demonstrated, although this response is low. In panel B
from figure 31 pCIM2 is compared to the control vector.

In this ELISA M2 protein expressed in insect cells was
used as coating (see Materials and Methods). A specific
anti-M2 response could be demonstrated, especially after
the third immunization. The higher anti-M2 response with
5 pCIM2 can be due to additional epitopes located in the
cytoplasmic domain of the M2 protein.

DISCUSSION

        The present document describes several systems
10 for the presentation of the highly conserved
extracellular part of the influenza A virus M2 protein to
the immune system. The M2 fragment was fused to the amino
terminus of the carrier protein in order to retain a free
N-terminus of the M2-domain and in this way mimic the
15 wild type structure of the M2 protein. The first fusion
protein, M2 linked to human ß2-microglobulin (IPM2hB2Mm),
was used to produce monoclonal antibodies. A second
fusion protein, M2 linked to Hepatitis B core protein
(IPM2HBcm) was used for vaccination studies. Both
20 proteins could also be used in the detection of a
specific antibody response against the extracellular part
of the M2 protein, since a correction has to be made for
antibodies directed against the carrier protein, which
are also produced during the immunization process.
25   The vaccination studies with IPM2HBcm showed
that the administered dose in the range that was used,
was apparently not a very critical parameter for
obtaining protection, as a dose ranging from 5 to 50 μg
protected the mice, although the immunized mice still
30 showed a high morbidity. This may have been due to the
high dose of virus (5 LD_{50}) that was used for the
challenge in order to obtain a clear-cut result for the
degree of protection. In a natural influenza infection
the number of infecting virus particles is much lower, so
35 that it can be assumed that the morbidity would decrease
accordingly.
        Analysis of the serum of immunized mice showed
a substantial antibody response towards the extracellular

part of the M2 protein, especially after viral challenge.
This latter, high response can be due to another way of
administration, intraperitoneal versus intranasal. Or it
can be explained on the basis of a more complete defense
5 mechanism against the incoming virus.

Slepushkin et al. (1995) described a
vaccination strategy, based on a membrane extract
containing the natural complete M2 protein for homologous
and heterologous virus challenge. But they used a very
10 strong adjuvant, incomplete Freund's, which is not
appropriate for medical use.

In contrast, the M2 extracellular domain
fusions of the invention described here can be obtained
in a pure form (at least 95% purity), and can be
15 administered in combination with safe adjuvants. A high
degree of protection was obtained, despite the fact that
the challenge was fairly severe. In view of the almost
invariant sequence of the M2 extracellular domain (see
table 1 which shows an overview of the amino acid
20 sequences of the extracellular domain of the influenza A
M2 protein) it may be expected that the protection
achieved will be similar against all human influenza A
strains known so far.

The vaccine may be further improved by the
25 inclusion of an influenza specific T helper epitope as
well as a CTL epitope into the fusion protein, for
example internally or linked to the C-terminus of the
Hepatitis B core protein. Other immunization routes are
possible as well, for example intraperitoneal versus
30 intranasal.

Besides the gram negative organism, E. coli,
also L. lactis was used, a gram positive organism, for
the expression of the M2HBcm fusion proteins. In L.
lactis it is not necessary to purify the expressed fusion
35 protein. The bacteria can be administered directly either
intranasally or orally.

A third promising carrier protein is also
described, namely the third complement protein fragment d

(C3d) (Dempsey et al., 1996). In a preferred
construction, three copies of the C3d protein are
preceded by the extracellular domain of the M2 protein.
This M2C3d3 fusion protein can be expressed either in an
5 intracellular form, anchored in the cell wall or secreted
into the growth medium, by genetic fusion to appropriate
regulatory sequences.

58

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CLAIMS

1. Influenza antigen, comprising a fusion product of at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and a presenting carrier.

5   2. Influenza antigen, wherein the presenting carrier is a presenting (poly)peptide.

3. Influenza antigen, wherein the presenting carrier is a non-peptidic structure, such as glycans, peptide mimetics, synthetic polymers.

10   4. Influenza antigen as claimed in claims 1-3 further comprising an additional domain for enhancing the cellular immune response immunogenicity of the antigen.

5. Influenza antigen as claimed in claims 1-4, wherein the conserved influenza membrane protein is the

15 M2 membrane protein.

6. Influenza antigen as claimed in claim 5, wherein the M2 membrane protein originates from influenza A virus.

7. Influenza antigen as claimed in claims 1-6,

20 wherein the presenting (poly)peptide is selected from the hepatitis B core protein, one or more C3d domains, tetanus toxin fragment C.

8. Influenza antigen as claimed in claims 1-7, wherein the antigen consists of Lactococci cells

25 expressing the fusion product in or on their cell membrane, optionally said cells release said product.

9. Influenza antigen as claimed in claims 1-8, wherein the functional fragment of the conserved influenza membrane protein is a fragment that is capable

30 of eliciting a statistically significant higher immunoprotection when administered in an immunoprotective dose to test members of a species than is found in control members of the same species not receiving the functional fragment.

10. Influenza antigen as claimed in claims 1-9, wherein the additional domain is an influenza specific T helper cell epitope or cytotoxic T cell epitope.

11. Influenza antigen as claimed in claims 1-10, obtainable by preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation regulatory sequences, bringing this gene construct in a suitable acceptor cell, effecting expression of the gene construct in the acceptor cell and optionally isolating the antigen from the acceptor cell or its culture medium.
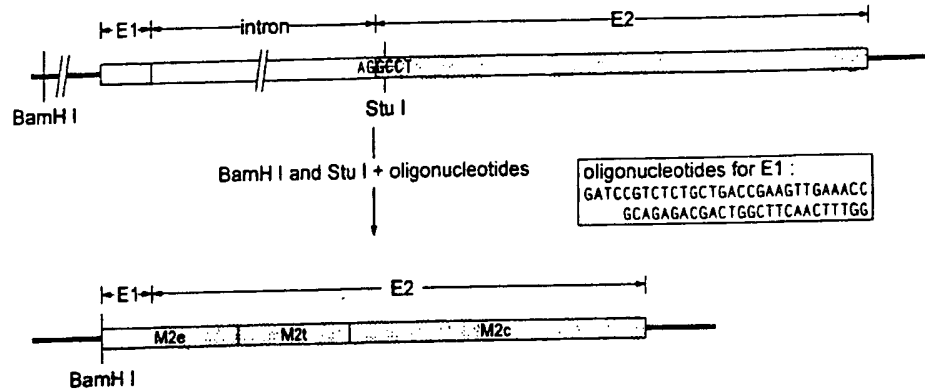
12. Influenza antigen as claimed in claim 11, wherein the coding sequence for the extracellular part of a conserved influenza membrane protein consists of a coding sequence for the extracellular part of the M2 protein of the influenza A virus or a functional fragment thereof and the coding sequence for the presenting (poly)peptide is selected from coding sequences for hepatitis B core protein, one or more C3d domains, or tetanus toxin fragment C.

13. Influenza antigen as claimed in claims 1-12, comprising the amino acids 2 to 24 of the M2 protein of influenza A virus, or modified versions thereof not substantially altering the tertiary structure of this part of the protein and hepatitis B core protein and/or one or more C3d domains.

14. Influenza antigen as claimed in claims 1-13 for use in the preparation of a vaccine against influenza for humans and animals.

15. Influenza antigen as claimed in claims 1-14 for use in the preparation of a vaccine against influenza A for humans and animals.

16. Vaccine against influenza, comprising at least an antigen as claimed in claims 1-15, optionally in the presence of one or more excipients.

17. Vaccine as claimed in claim 16, wherein the antigen is in isolated form.

18. Vaccine as claimed in claim 16, wherein the antigen is part of a membrane fragment.

19. Vaccine as claimed in claim 16, wherein the antigen is anchored in the membrane of an acceptor cell expressing the antigen.

20. Vaccine as claimed in claim 16, wherein the antigen consists of Lactococci cells expressing the fusion product in or on their cell envelope.

21. Vaccine as claimed in claims 16-20, further comprising one or more other influenza antigens, for example selected from hemagglutinin, neuraminidase nucleoprotein and/or native M2.

22. Use of an antigen as claimed in claims 1-13 for the preparation of a vaccine against influenza.

23. Method of preparing an antigen as claimed in claims 1-15, comprising the steps of:

a) preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation regulatory sequences,

b) bringing this gene construct in a suitable acceptor cell,

c) effecting expression of the gene construct in the acceptor cell, and

d) optionally isolating the antigen from the acceptor cell or its culture medium.

24. Acceptor cell, expressing an antigen as claimed in claims 1-15.

25. Acceptor cell as claimed in claim 24, wherein the cells are Lactococcus cells.

Figure 1

A


```
       |-E1-|+—— intron ———+|——————————— E2 ———————————|
  ╫┤╫         ╫              AGGCCT
  BamH I                     Stu I
                              |
              BamH I and Stu I + oligonucleotides      ┌─────────────────────────────────────┐
                              |                         │ oligonucleotides for E1 :            │
                              ↓                         │ GATCCGTCTCTGCTGACCGAAGTTGAAACC       │
                                                        │   GCAGAGACGACTGGCTTCAACTTTGG         │
                                                        └─────────────────────────────────────┘

       |-E1-|+————————————— E2 —————————————|
  ▬▬▬▬▬[  M2e  | M2t |        M2c        ]▬▬▬▬▬
  BamH I
```

B


```
       |——— M2e ———+—— M2t ——+———— M2c ————|
  ▬▬▬▬▬[  TGATCC                          ]▬▬▬▬▬
  BamH I
                              |
              Site-directed mutagenesis              ┌─────────────────────────────────────┐
              (Stanssens et al., 1989)               │ mutator oligonucleotide :            │
                              |                       │ CGGTTCAAGTGATCATCTCGC                │
                              ↓                       │              Bcl I                   │
                                                      └─────────────────────────────────────┘
  ▬▬▬▬▬[  TGATCA                          ]▬▬▬▬▬
  BamH I   Bcl I
```
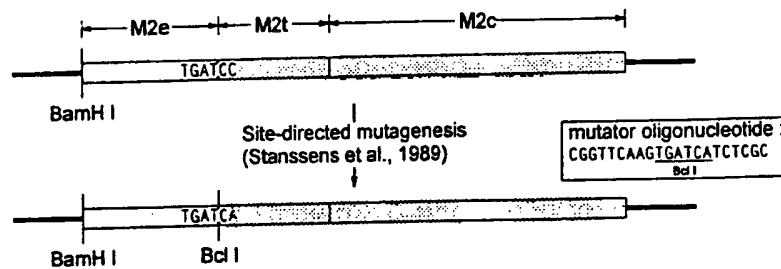
C

	2	3	4	5	6	7	8	9	10	11
Nucleotide sequence :	TCT	CTG	CTG	ACC	GAA	GTT	GAA	ACC	CCT	ATC
Amino acid sequence :	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile

	12	13	14	15	16	17	18	19	20	21	22	23	24
	AGA	AAC	GAA	TGG	GGG	TGC	AGA	TGC	AAC	GGT	TCA	AGT	GAT
	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Gly	Ser	Ser	Asp
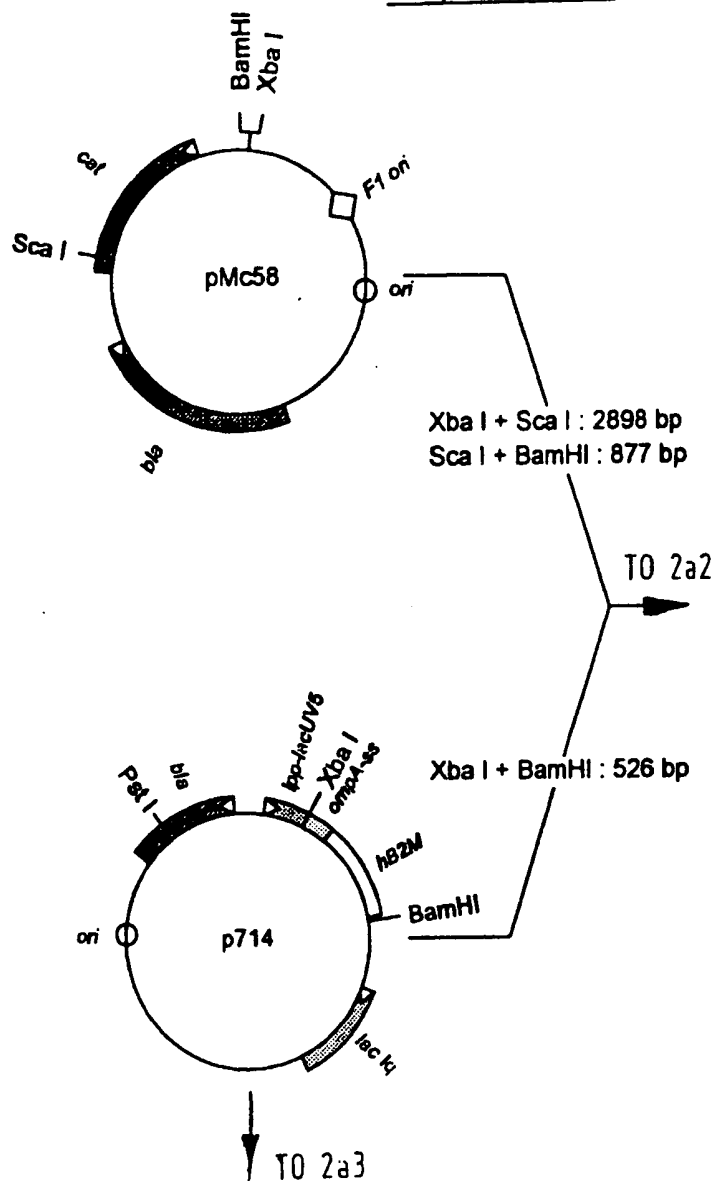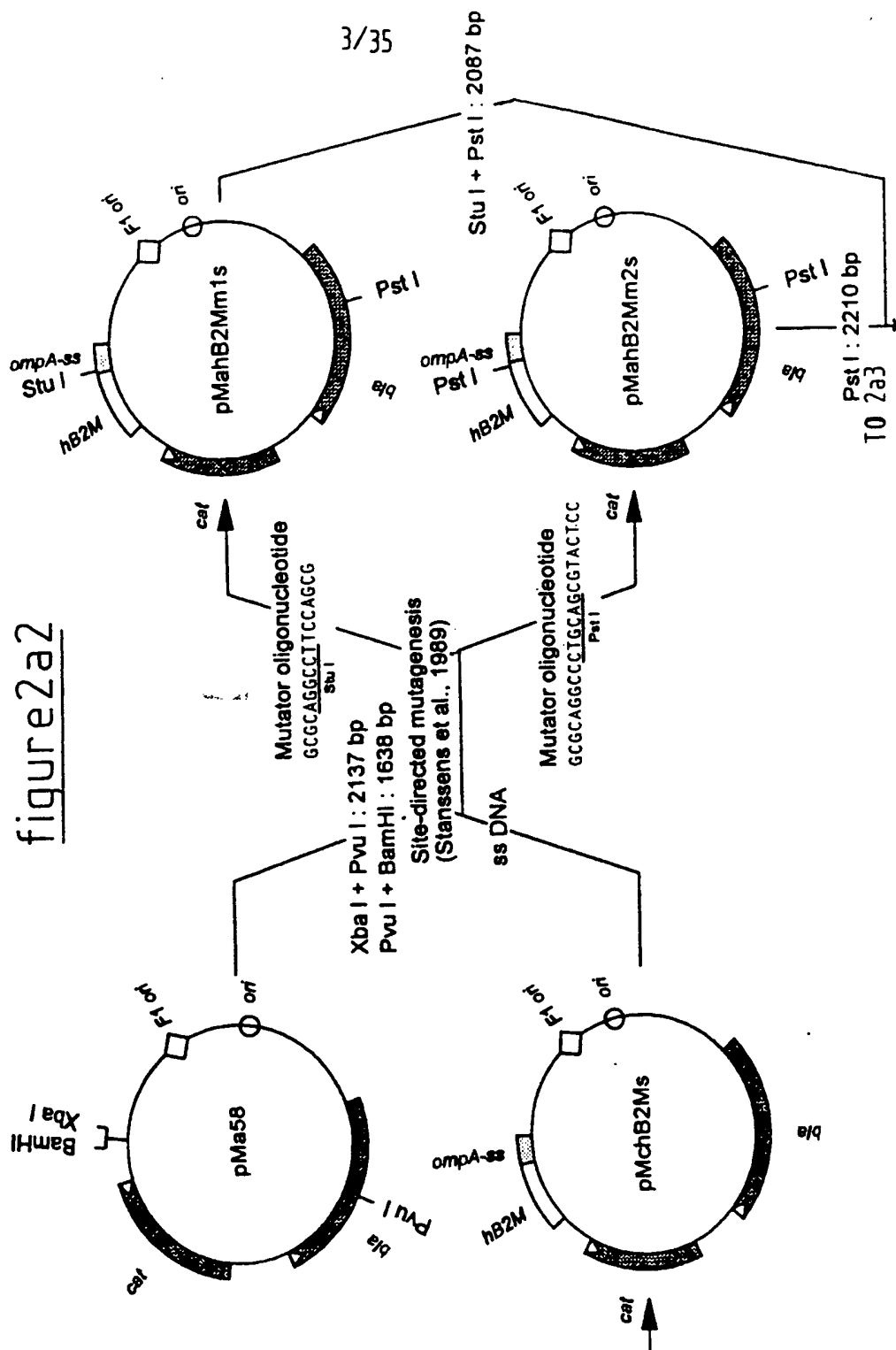
figure 2a1

figure2a2
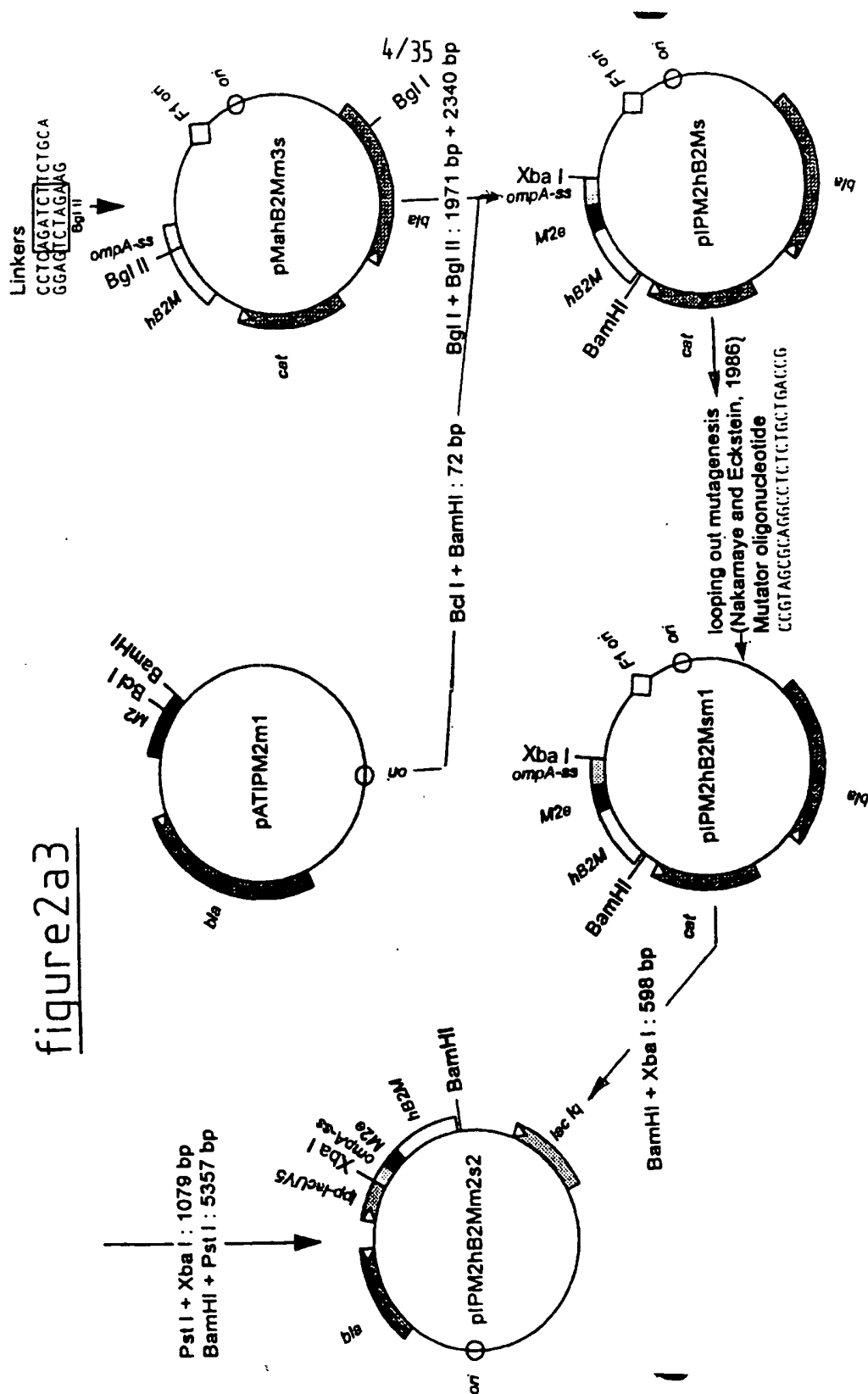
figure2a3

Figure 2b

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figure 3a1

pMa58

EcoRI
Sma I
cat
Sca I
Ban II
F1 ori
Ban II
ori
Sca I
bla
Bgl I

Bgl I + Sma I : 1886 bp
Sma I + Bgl I : 1917 bp
Nco I linker : GCCATGGC

pMa581

cat
Nco I
Xba I
F1 ori
ori
bla
Pst I

Pst I + Nco I : 1763 bp
Xba I + Pst I : 2011 bp

TO 3a2
TO

Nco I + Xba I : 346 bp

pPLcA1

Nco I
HBc
Xba I
cat
bla
ori

Sca I + Nco I : 1052 bp
Xba I + Sca I : 1800 bp

TO.3a3

EcoRI + Sca I : 2286 bp

Sma I + Ban II + Klenow : 990 bp

TO. 3a4

TO 3a3

figure3a2

figure 3a3

figure3a4

pMalPM2HBc2

Stu I + Hind III :
586 bp + 2843 bp

looping out mutagenesis
(Deng and Nickoloff, 1992)
Mutator oligonucleotide :
CGGTCAGCAGAGACATGGGTAATCC
Selection oligonucleotide
CCAGACCGTTCAGCTGGATATTACGG

pIPM2HBc2m

SgrAI + EcoRI : 499 bp

T03a3

Figure 3b

Figure 3c

Figure 4

Figure 7

Figure 5

Figure 6

ATG	TCT	CTG	CTG	ACC	GAA	GTT	GAA	Nucleotide sequence of *ipm2hbcm*
Met	Ser	Leu	Leu	Thr	Glu	Val	Glu	Translated amino acid sequence
	Ser	**Leu**	**Leu**	**Thr**	**Glu**	**Val**	**Glu**	Amino terminus of the fusion protein IPM2HBcm
	Ser	Leu	Leu	Thr	Glu	Val	Glu	Amino terminus of the M2 protein of A/Udorn/72
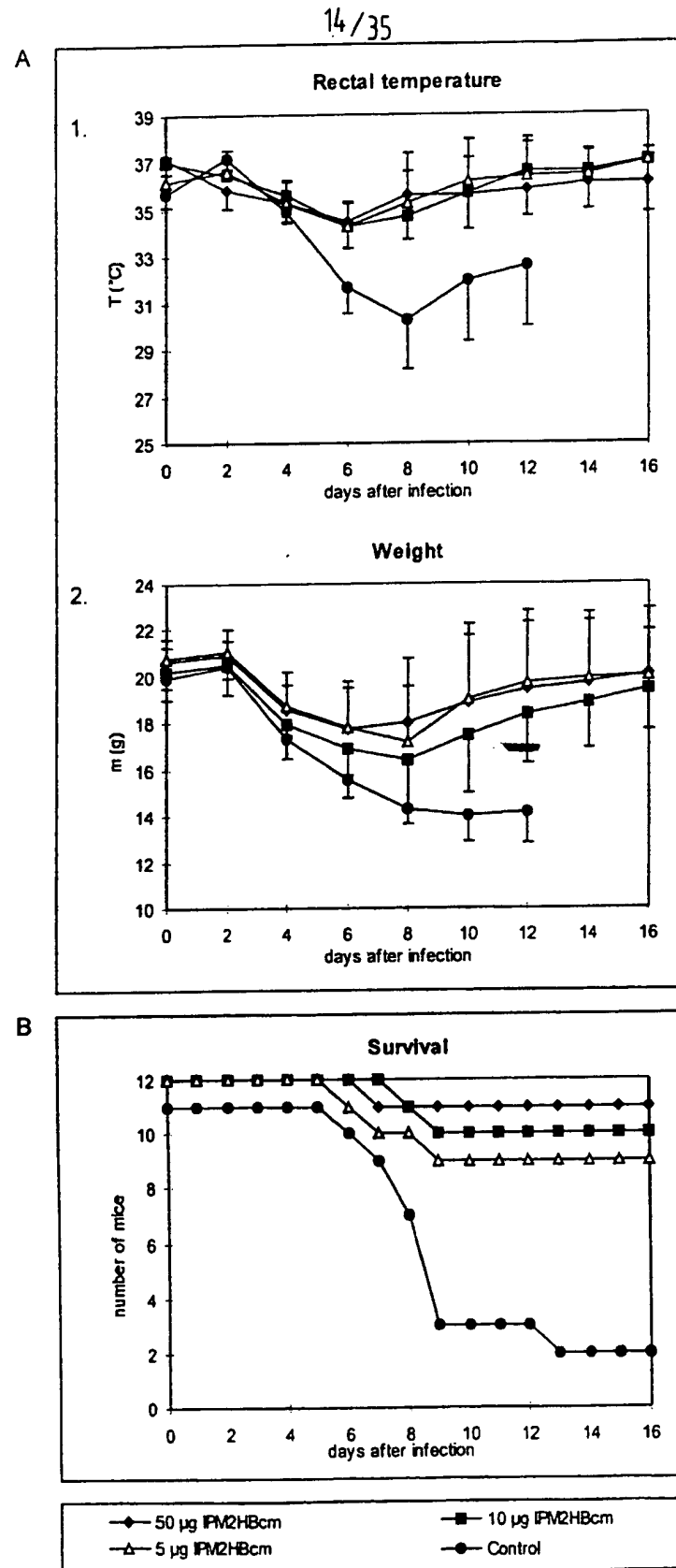
Figure 8

fig.8D
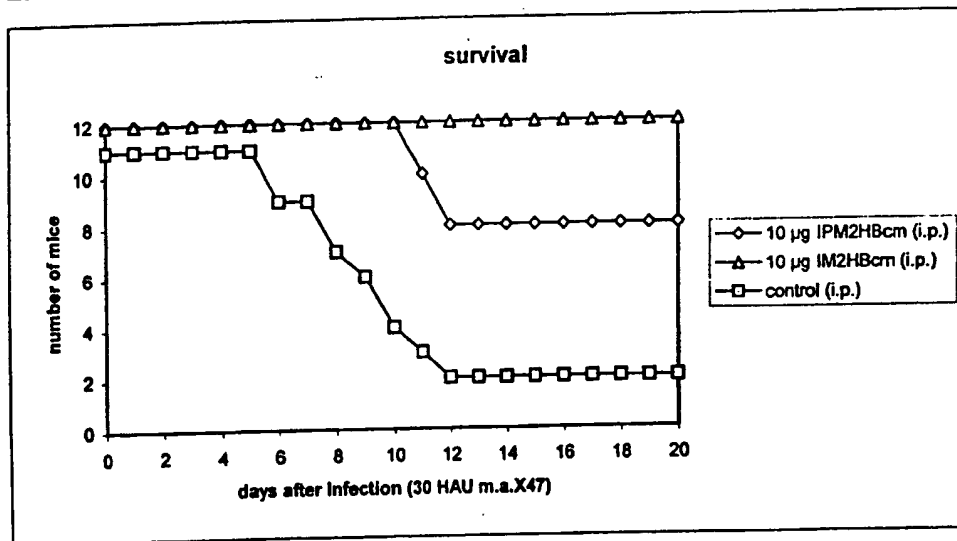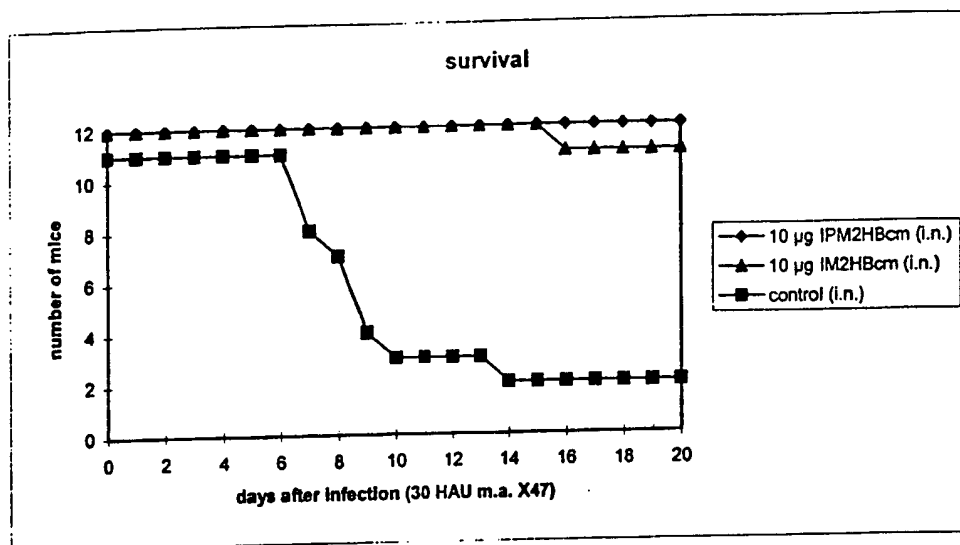
fig.8C

	surviving mice
10 μg IPM2HBcm (i.n.)	12/12
10 μg IM2HBcm (i.n.)	11/12
control (i.n.)	2/11
10 μg IPM2HBcm (i.p.)	8/12
10 μg IM2HBcm (i.p.)	12/12
control (i.p.)	2/12
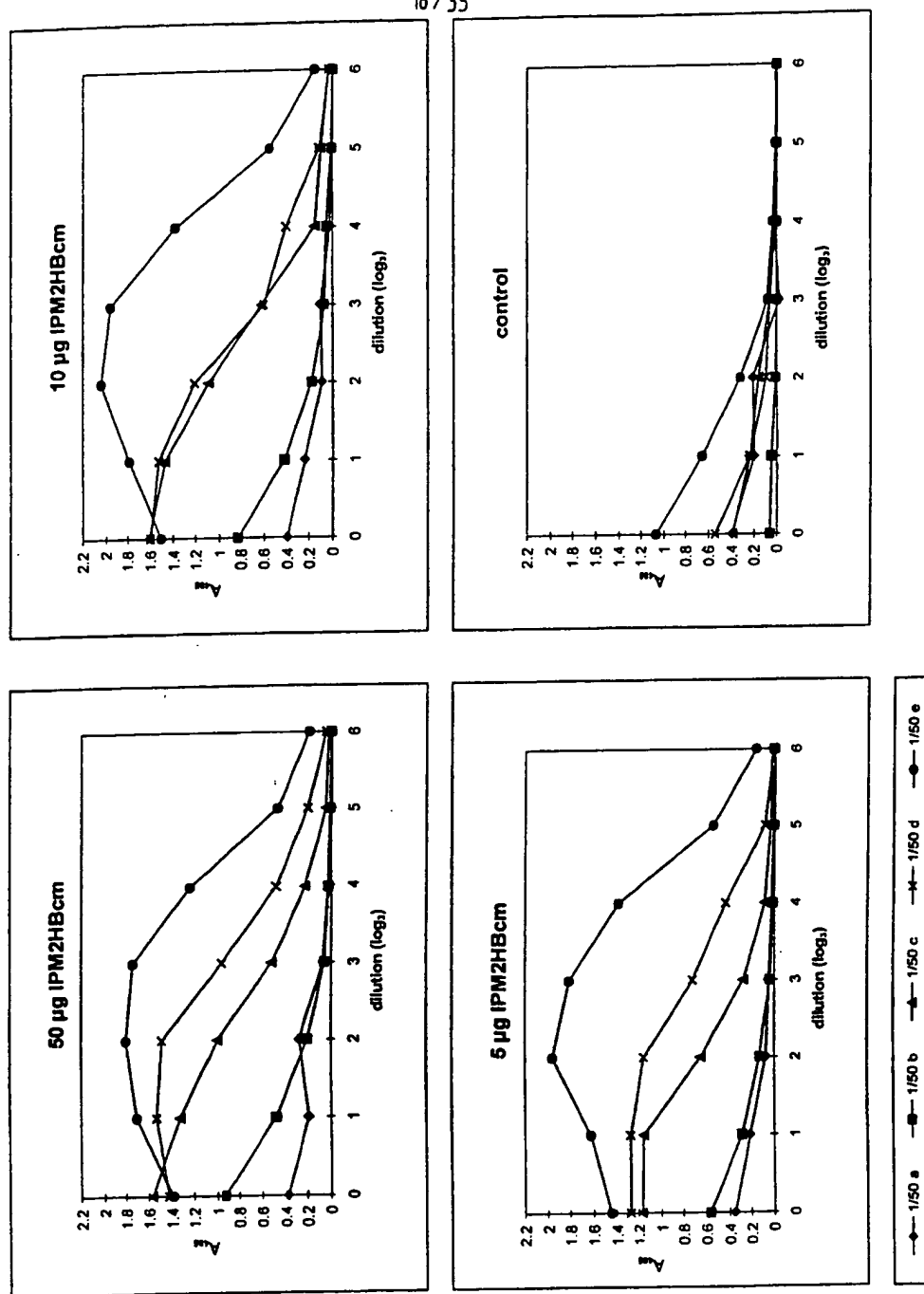
Figure 9

Figure 10   17 / 35
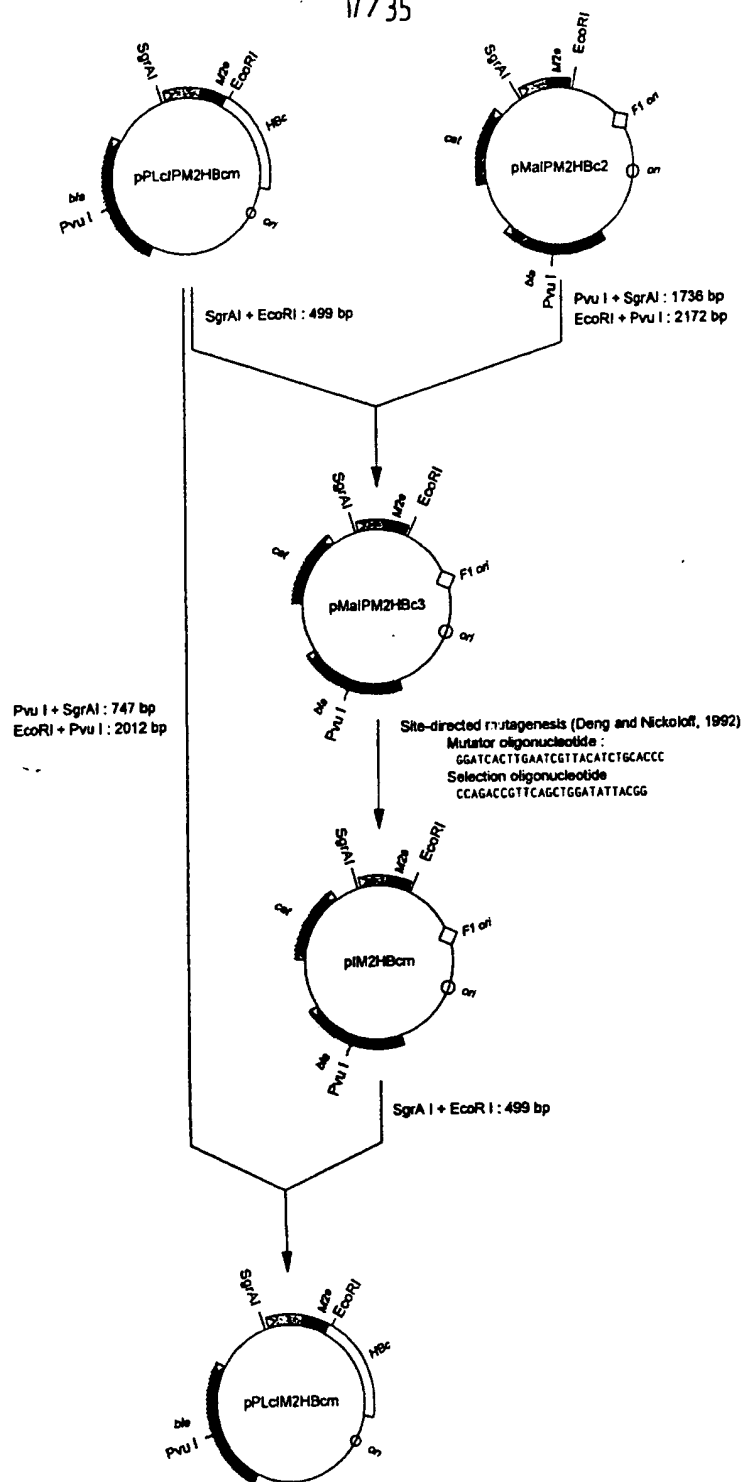
Figure 11
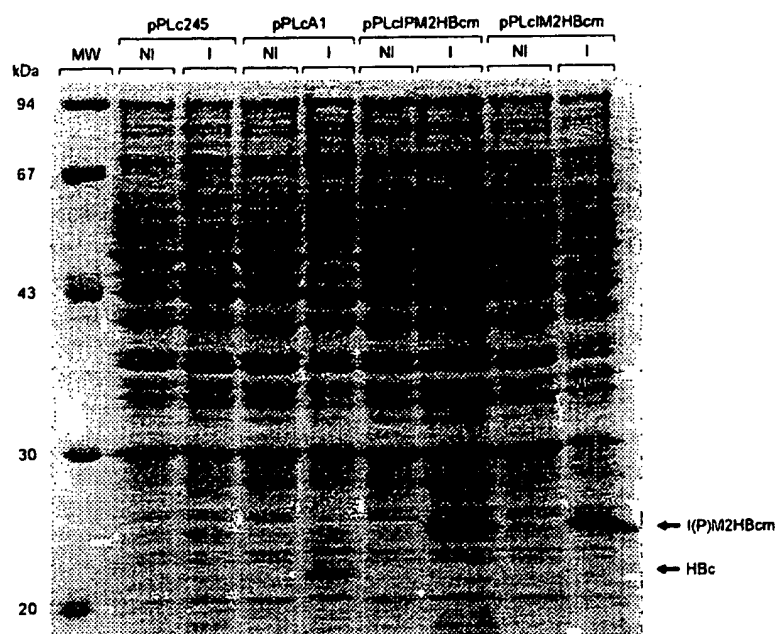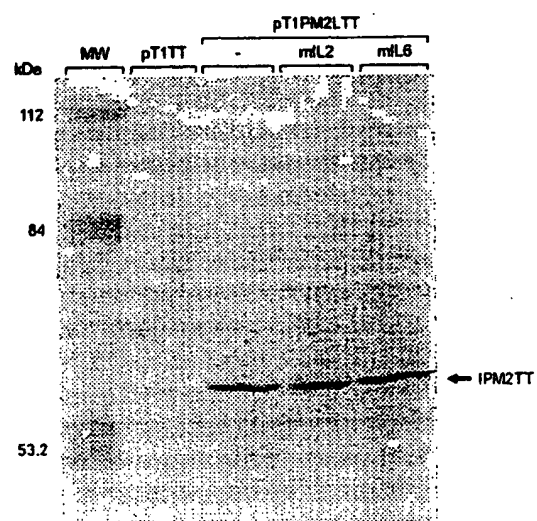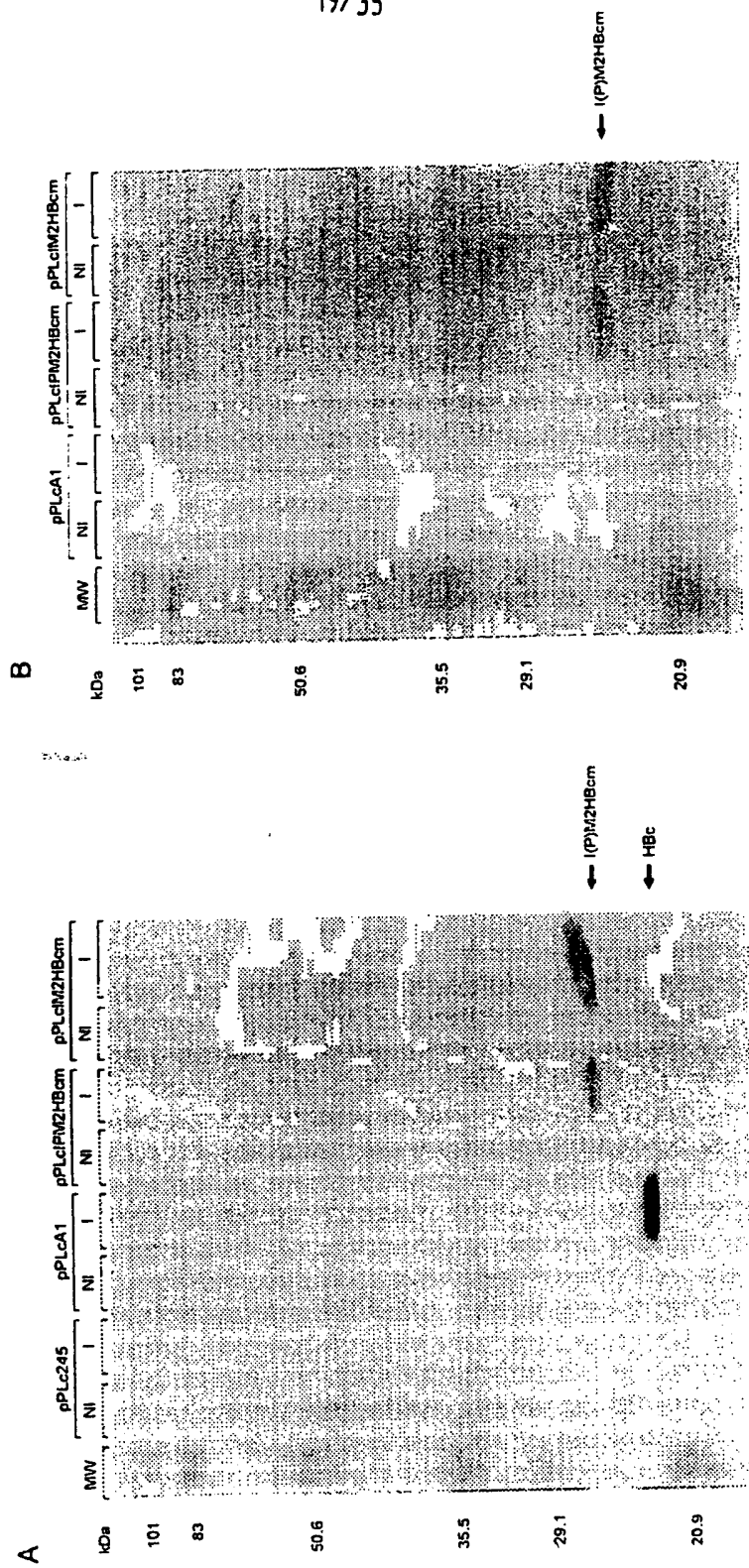
Figure 21

Figure 12

Figure 13   20/35

HBcs (27-mer)
  C<u>ATG</u>GATATGGATCCTTATAAAGAATT
    start

M2s (23-mer)
  C<u>ATG</u>TCTCTGCTGACCGAAGTTG
    start

M2Ls (29-mer)
  C<u>ATG</u>TCT TTATTA ACCGAAGTTGAAACCC
    start

HBca (39-mer)
  CG<u>TGATCA</u>AC<u>TAGT</u>TCACTAACATTGAGATTCCCGAGAT
    Bcl I   Spe I stop

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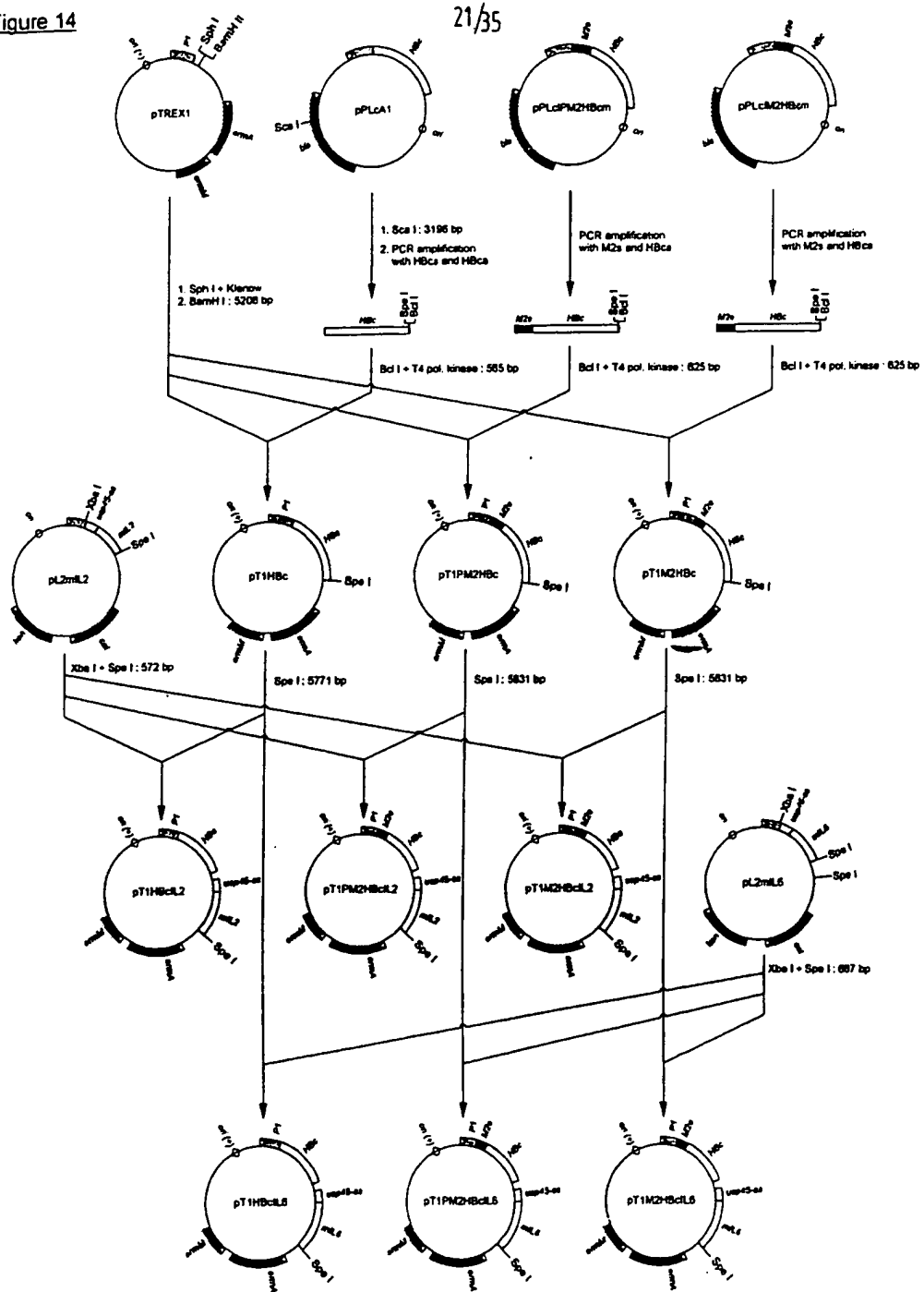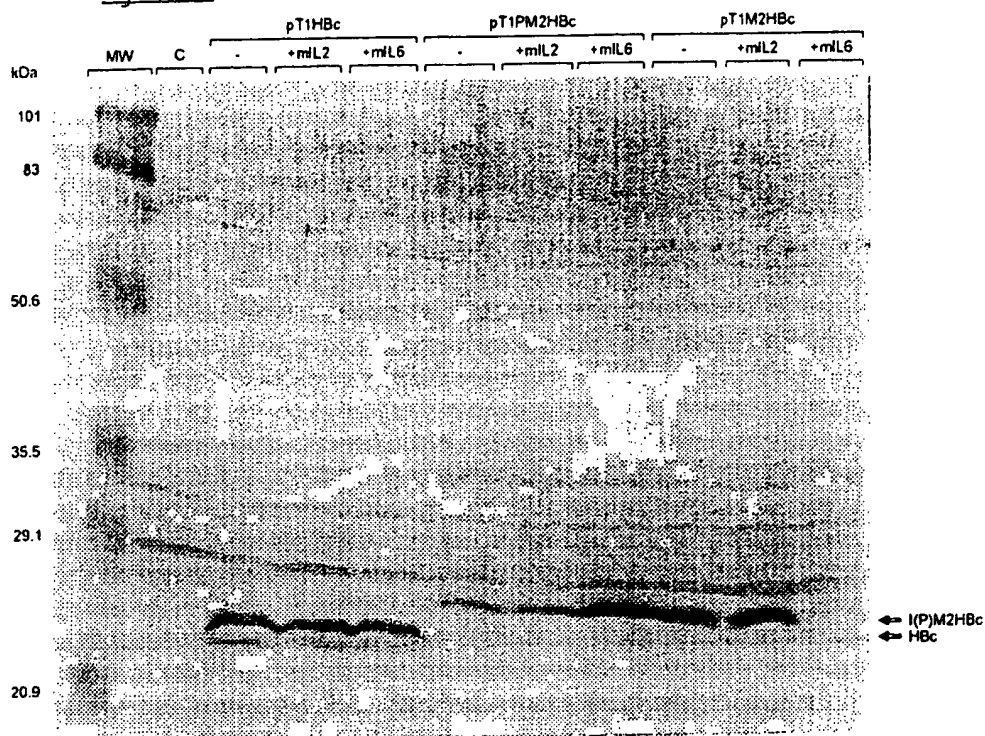
Figure 14

Figure 15

Figure 16
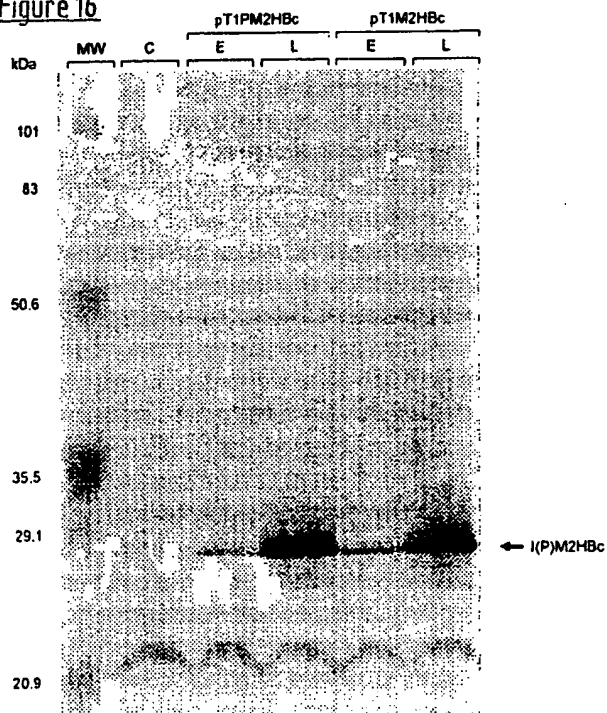
Figure 17

M2Ca (33-mer)

CG<u>GGATCC</u>CCACTTGAATCGTTACATCTGCACC
 <u> BamH I </u>

M2LSs (30-mer)

<u>TCTTTTATTA</u>ACCGAAGTTGAAACCCCTATC
 Ser

C3ds (35-mer)

CCGCGCCCACCCGACGA<u>GATCT</u>CGGATCTACCCCC
 <u>Bgl II</u>

C3da (38-mer)

GC<u>ACTAGT</u>TCAA<u>GGATCC</u>GATCCGAACTCTTCAGATCC
  Spe I   stop   BamH I

Figure 18   24/35
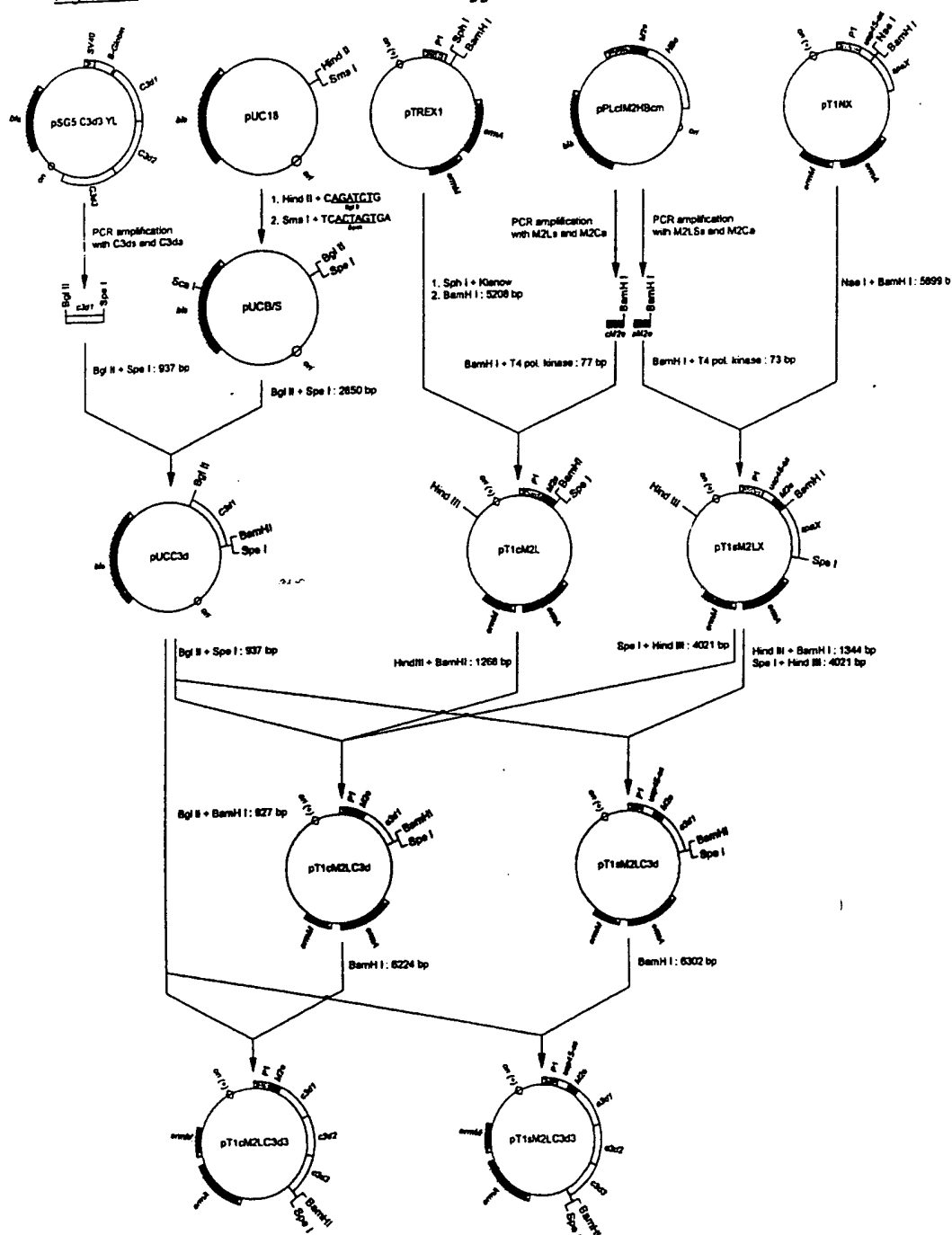
Figure 19   25/35

TTFCs (35-mer)

CG<u>GGATCC</u>GACACCAATTCCATTTTCTTATTCTAA
    BamH I

TTFCa (25-mer)

GG<u>GGATCC</u><u>ACTAGT</u><u>TTA</u>ATCATTTG
   Bcl I   Spe I stop

M2Ls (29-mer)

C<u>ATG</u>TCT TTATTA ACCGAAGTTGAAACCC
 start

Figure 20
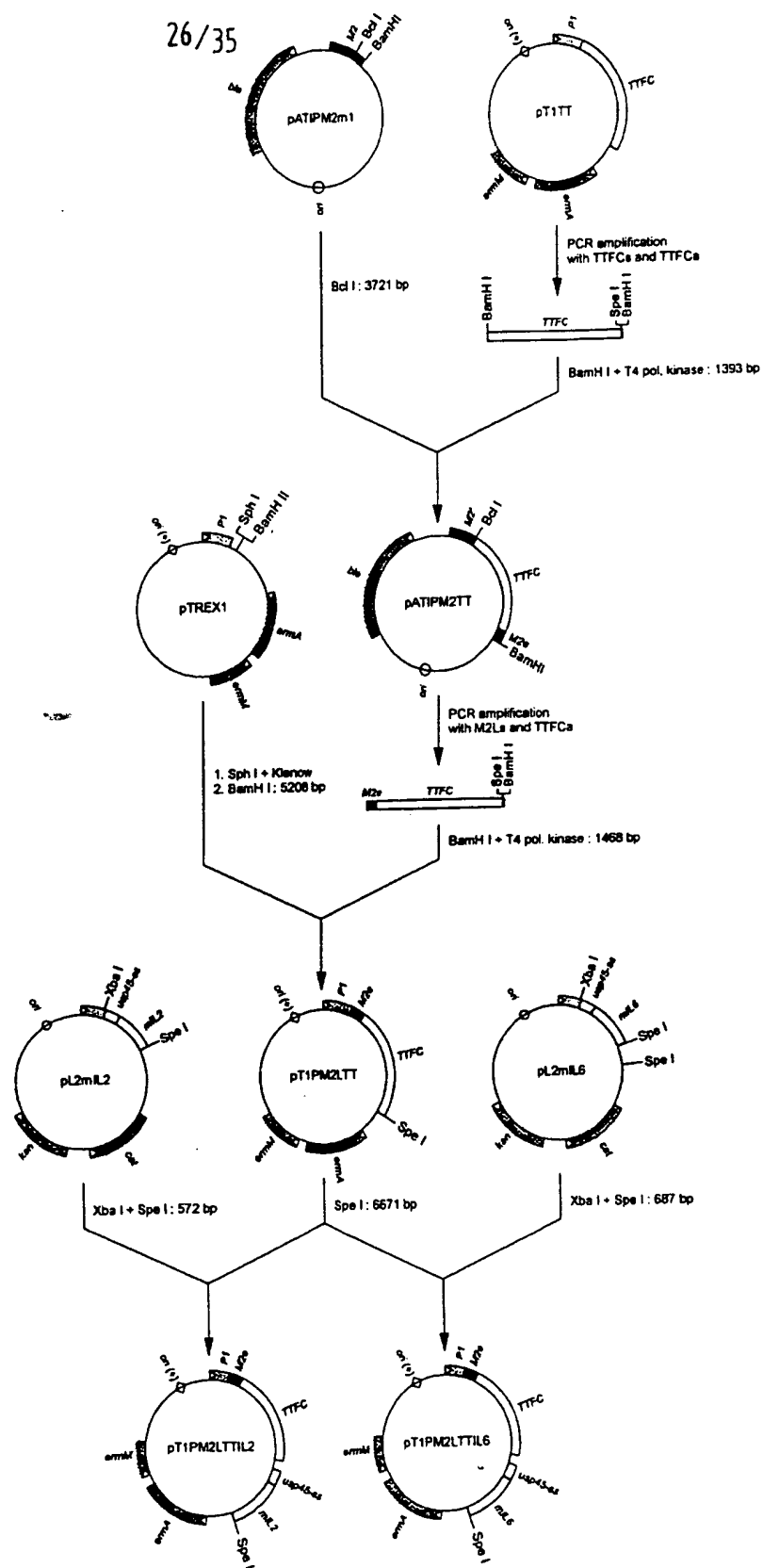
Figure 22

GP67s (25-mer)
GCT<u>ACTAGT</u>AAATCAGTCACACCAA
    SpeI

GP67a (33-mer)
CG<u>AAGCTT</u><u>GCCGGC</u>AAAGGCAGAATGCGCCGCC
   HinDIII   NaeI

Figure 23

M2Ss (23-mer)
TCTCTGCTGACCGAAGTTGAAAC

UM2ECa (50-mer)
CG<u>AAGCTT</u><u>ACTAGT</u>TCA<u>CGGATCC</u>CCACTTGAATCGTTGCATCTGCACCC
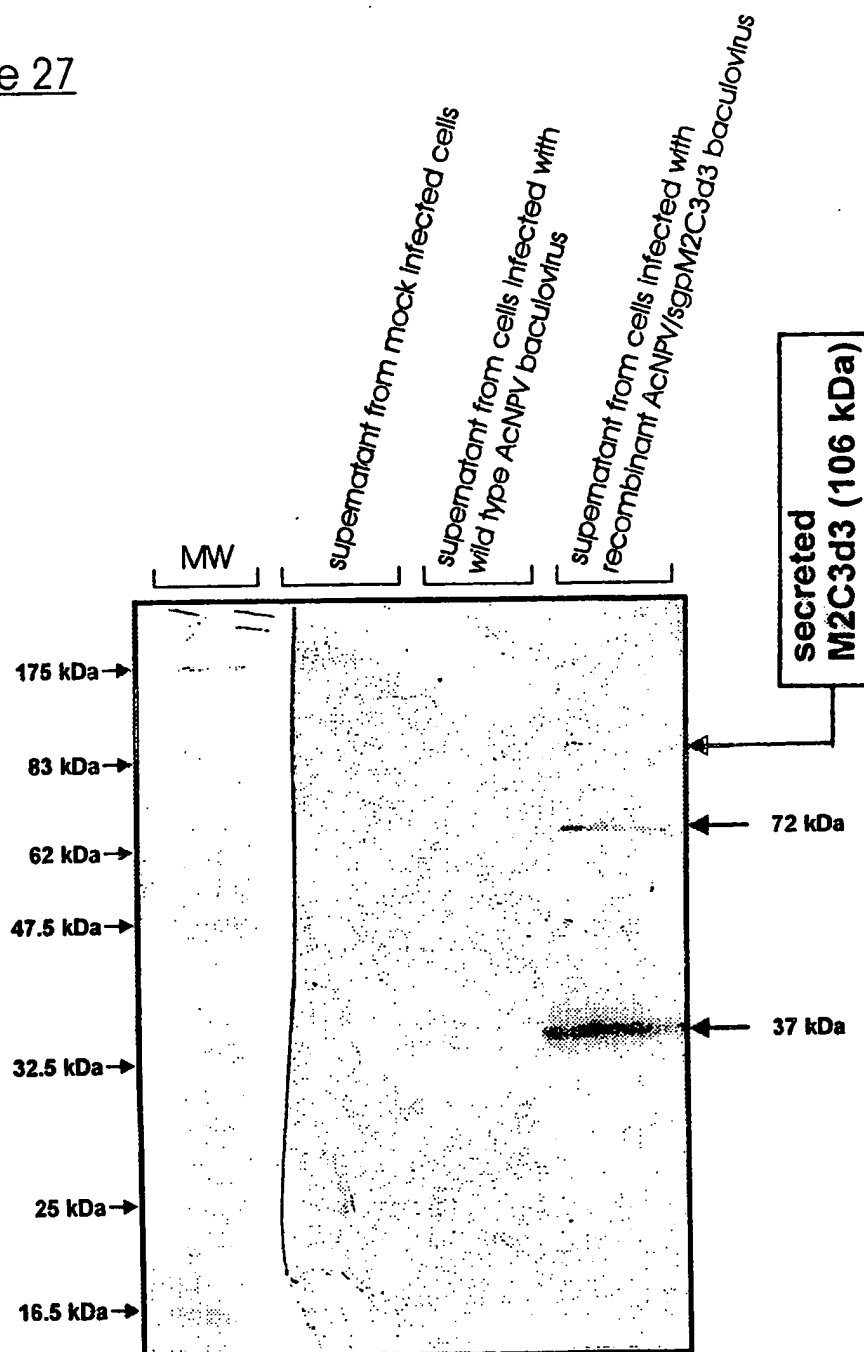 HindIII   SpeI   stop   BamHI

Figure 24

Figure 25

Figure 26

Figure 27

Figure 28

Figure 29

Figure 30

Figure 31   35/35